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"Molecular cloning and expression analysis of genes involved in the compensatory growth of sea bass *(Dicentrarchus labrax)*"

Ph.D. student: Samuela Corà

Tutor: Dott.ssa Genciana Terova

Index

Summary

1. Introduction ..... pag. 1

1.1 Compensatory growth in vertebrates ..... pag. 5

1.1.1 Characteristics of compensatory growth ..... pag. 5

1.1.2 Changes in body composition during compensatory growth ..... pag. 7

1.2 Compensatory growth in fish ..... pag. 9

1.2.1 Methodological studies and factors affecting compensatory growth ..... pag. 9

1.2.2 Proximate causes of compensatory growth ..... pag. 12

1.2.3 Modelling compensatory growth ..... pag. 13

1.2.4 Proximate fish composition during food deprivation and recovery ..... pag. 14

1.3 Endocrine regulation of fish growth ..... pag. 16

1.4 Control of appetite in fish ..... pag. 20

1.5 Fish nutritive physiology during compensatory growth ..... pag. 23

1.6 Dietary lipid requirements and effect of nutritional status on fish ..... pag. 25

1.6.1 Genes involved in fatty acid metabolism: Δ6 desaturase, lipin and PPAR ..... pag. 27

1.7 Protein and amino acid requirements and effect of nutritional status on fish ..... pag. 32

1.7.1 The oligopeptide transporter 1, PepT1 ..... pag. 33

1.8 Project purpose ..... pag. 36

2. Materials and methods ..... pag. 38

2.1 Animals and feeding protocol ..... pag. 38

2.2 Preparation of total RNA and cDNA synthesis ..... pag. 39

2.3 Cloning and sequencing ..... pag. 40

2.4 5' and 3' RACE analysis ..... pag. 40

2.5 Quantitative real-time RT-PCR ..... pag. 45
2.5.1 Generation of in vitro-transcribed cRNAs for standard curves ..... pag. 45
2.5.2 Generation of standard curves ..... pag. 47
2.5.3 Quantitation of transcripts by one-step RT-PCR TaqMan system ..... pag. 47
2.5.4 Sample quantification ..... pag. 48

2.6 Calculation and statistical analysis ..... pag. 49

2.7 Sea bass PepT1 in silico analysis ..... pag. 49

3. Results ..... *pag. 50*

3.1 DNA sequencing and bioinformatic analysis ..... pag. 50

3.1.1 Δ6 desaturase cDNA sequence ..... pag. 50
3.1.2 Lipin cDNA sequence ..... pag. 53

3.2 Sea bass PepT1 cDNA sequence ..... pag. 55
3.2.1 Sea bass PepT1 phylogenetic analysis ..... pag. 59

3.3 Fish growth and feeding rates ..... pag. 61

3.4 Creation of standard curves for absolute quantification of genes target ..... pag. 63

3.5 mRNAs copy number of genes involved in lipid metabolism in sea bass, during fasting and refeeding ..... pag. 64
3.5.1 Δ6 desaturase ..... pag. 64
3.5.2 Lipin and PPARγ ..... pag 66

3.6 PepT1 mRNA copy number in sea bass tissues ..... pag. 69

3.7 PepT1 mRNA copy number in sea bass proximal intestine during fasting and refeeding ..... pag. 72

4. Discussion ..... *pag. 73*

4.1 Conclusion ..... pag. 85

5. References ..... *pag. 86*

Summary

In natural aquatic systems, many fish species experience periods of fasting during their life cycle because of the high variation in food availability. Although fish are highly tolerant to relatively long periods of food deprivation, energy storage and growth rate is reduced in those that experience different degrees of starvation, redirecting this energy towards maintenance metabolism. Fasting-associated growth retardation is completely overcome, or at least reduced, if an abundant food supply becomes available after a prolonged period of food shortage. Then, fish display an exceptionally rapid growth rate known as "compensatory growth".

There are several descriptions of compensatory growth in fishes, but the mechanisms involved in such rapid recovery from fasting are still not fully understood. Such mechanisms have principally been searched for at the level of total fish growth where a number of circulating hormones are thought to be involved. Some attention has also been paid to muscle growth and to locally produced paracrine/autocrine factors, which can profoundly affect tissue growth and development. Only little information is available at the molecular level.

Accordingly, this research focused on identifying candidate genes whose expression contributes to the compensatory growth induced by refeeding in sea bass (*Dicentrarchus labrax*), a marine fish of great interest for Mediterranean aquaculture. In the course of the study, we firstly isolated the complete cDNAs encoding diverse physiologically relevant proteins involved in lipid and protein metabolism, and then assessed the impact of chronic feed deprivation and subsequent refeeding on their mRNA abundance levels in different tissues, with the aim to relate these expression levels to fish feeding status.

The molecular cloning and sequencing strategy resulted in the isolation of cDNAs that encode the following proteins:

- Δ6 desaturase, which has a recognized capacity to desaturate the fatty acids with 18 carbon atoms, into highly unsaturated fatty acids (HUFA) with 20 and 22 carbon atoms.
- Lipin, an enzyme required for triacylglycerol and phospholipid biosynthesis, and a transcriptional coactivator in regulating lipid metabolism genes.

- Peroxisome proliferator-activated receptor (PPARγ), a central factor in the control of the expression of genes involved in lipid homeostasis.
- Oligopeptide transporter (PepT1), an integral plasma membrane protein responsible for the uptake of dietary di- and tri-peptides from the intestinal lumen into the enterocytes.

The isolated sequences were then deposited in GenBank databases with the following accession numbers: EU647692 for Δ6 desaturase; EU644089 for lipin; FJ237043 for PepT1. The evolutionary relationship of all target genes with respect to other publicly available related genes in other teleosts, amphibian, avian, and mammalian species, was also studied.

After the sequence isolation, for the quantification by real-time RT-PCR of each gene transcript levels, in response to the nutritional status of the animals, a fasting and refeeding trial was conducted at the marine water recirculating system at our department in Varese. Briefly, 140 sea bass were stocked into four tanks of 2 m^3 each, with 35 fish per tank. At the start of the experiment, all the fish were weighed, and two of the tanks were randomly assigned to each of two treatments. Fish in these two tanks were fed to apparent satiety (control), whereas fish in the other two tanks were deprived of food for 35 days and then refed to apparent satiety for 21 days with the same type of feed utilized before fasting. Five fish from each of the experimental groups were sampled at the following time points: before fasting (day 0), 4 days after fasting, at the end of fasting, and then sequentially at 4, 14, and 21 days following refeeding. For the molecular biology analysis, the whole digestive tract, liver, gill, heart, kidney, ovary, brain, muscle and spleen were dissected out.

After 35 days of fasting body weight of fasted fish were lower than the fed controls, whereas during the subsequent refeeding period, previously unfed fish were able to increase body weight sufficiently to overcome weight loss imposed by the 35 days feed restriction. This is the evidence that the compensatory growth was in play.

The results of the real-time RT-PCR absolute quantification using the mRNA standard curve method, revealed that the nutritional status significantly influenced Δ6 desaturase, lipin and PPARγ mRNA copy number in the liver of sea bass, inducing an up-regulation during prolonged fasting (35 days) and a down-regulation during the recovery from fasting (21 days of refeeding).

In the proximal intestine, 35 days of fasting contributed to a significant decrease in Δ6 desaturase transcript levels, whereas the subsequent recovery from fasting was associated with an increase in Δ6 desaturase mRNA copy number, which return to normal (control) levels after 21 days of refeeding.

The abundance of lipin and PPARγ mRNA levels in the proximal intestine were not affected by the availability of food, as they remained similar between fed, fasted and refed fish.

The nutritional status significantly influenced PepT1 mRNA copy number in the proximal intestine of sea bass, inducing a down-regulation during 35 days of fasting and an up-regulation during the subsequent 21 days of refeeding. This pattern of expression seems to be in complete support of the compensatory growth. The increase in the transcripts of the intestinal oligopeptide transporter Pept1 might be correlated to the great quantity of protein taken in with the food in the initial days of refeeding. In fact, refeeding of sea bass after a long starvation period was marked by hyperphagia, as early as the first day.

In conclusion, we have isolated the cDNA sequences encoding Δ6 desaturase, lipin, PPARγ and Pept1in sea bass, and also demonstrated that the nutritional state of the animal influences their levels of expression. The present study is the first one to investigate the behavior of these transcripts over a long period of fasting and subsequent refeeding of fish. We recognize that mRNA levels in our study do not measure physiological effects produced by the proteins. Due to this, our hypothesis that the aforementioned genes are important triggers of the fasting response in the sea bass, will have to be confirmed by future studies.

1. Introduction

The word "aquaculture", although used quite widely for the last two decades to denote all forms of cultivating aquatic animals and plants in fresh, brackish and marine environments, is still defined by many individuals in a more restrictive sense. For some, it means aquatic culture other than fish farming or fish husbandry, whereas other people understand it as aquatic farming other than mariculture. However, the term aquaculture is sufficiently expressive and all-encompassing (Pillay and Kutty, 2005).

After growing steadily, particularly in the last four decades, aquaculture is for the first time set to contributed half of the fish consumed by the human population worldwide. This reflect not only the vitality of the aquaculture sector but also global economic growth and continuing developments in fish processing and trade (FAO, 2009).

Aquaculture takes a variety of forms: extensive or intensive, in natural settings or tanks, in fresh water or sea water, in flow-through or recirculation systems, traditional or modern, and so on. Traditional, extensive fish farming consists in maintaining ponds (natural or artificial) and lagoons in such a way that they foster the development of aquatic fauna, particularly promoting/encouraging the growth of juveniles/fry until they reach a "marketable" size at a higher yield than that of the natural ecosystem. Traditional, combined forms of fish farming in ponds and lagoons have increasingly evolved more into managed production modes described as semi-extensive aquaculture. This assists nature by introducing fry from hatcheries into the sites and providing supplemental feed.

For a yield much greater than semi- and extensive aquaculture provide, an intensive fish farming site is used, generally composed of several tanks of different sizes and depths suited to the different stages of growth of the fish, either floating at sea or in permanent structures on land. In such installations, water recirculation system are applied which offer the advantages of isolation from the external environment, meaning that all the parameters of the water can be controlled: temperature, acidity, salinity, disinfection, etc. Moreover, to achieve a greater production it is necessary to introduce from outside all that the natural system is

unable to supply to support a greater biomass: food, oxygen and removing catabolites.

Among these fish farming systems, the role of saltwater species aquaculture is undeniably significant as marine fish represent one of the most important sources of animal protein for both animal and human health.

In the 1960s, the floating cage was developed as a major innovation in sea farming and its availability at a reasonable price proved to be an unprecedented commercial success and turned sea farming into an up-and-coming sector in Europe.

The Mediterranean countries studied and developed the rearing of sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) with this system, and, during the 1990s, farming of these species spread throughout the Mediterranean. Salmon, sea bass, and seabream remain the flagship products of European sea farming, offering a diversification in quality that appeals to different market segments.

The 1990s and the first decade of the new century saw the development of another form of intensive sea farming, this time of flatfishes: inland tanks supplied with sea water were therefore introduced, and progress in recirculation technology offered new prospects for land-based mariculture. The start of the 21st century nevertheless has brought a sizeable new challenge for aquaculture. European coastal zones are saturated with activity and have no more land to offer for expanding aquaculture. Sea farming is obliged to move further away from the coast. It can move inland, thanks to recirculation systems, but the cost of artificial reconstitution of sea water represent a disadvantage. It can also move off-shore, far from sheltered coastal areas. Off-shore mariculture is the new field of research in European aquaculture.

According to FAO statistics, capture fisheries and aquaculture supplied the world with about 110 million tonnes of food fish in 2006, providing an apparent per capita supply of 16.7 kg (live weight equivalent) which is among the highest on record; on this total, aquaculture accounted for 47 percent (FAO, 2009). Overall, fish provided more than 2.9 billion people with at least 15 percent of their average per capita animal protein intake: the share of fish proteins in total world animal protein supplies grew from 14.9 percent in 1992 to a peak of 16 percent in 1996, declining to about 15.3 percent in 2005. In 2006, 77 percent of world fish production was

used directly for human consumption and almost of the remaining 33 million tonnes was destined for non-food products, in particular the manufacture of fishmeal, and fish oil (FAO, 2009).

Fifty years ago, many people believed that marine fisheries were inexhaustible. Half a century later, after a devolution in the industry and rapid development in science and technology, modernistic fish-detecting devices and fishing techniques have left fish with no place to hide. We would all agree that fishery resources are depleting mainly due to anthropogenic factors such as overfishing, habitat destruction, pollution, introduction of invasive species, and climate change. An overall review of the state of marine fishery resources confirms that the proportions of overexploited, depleted and recovering stocks have remained relatively stable in the last 10-15 years. In 2007, about 28 percent of stocks were either overexploited (19 percent), depleted (8 percent) or recovering from depletion (1 percent) and thus yielding less than their maximum potential owing to excess fishing pressure (FAO, 2009).

In order to optimize production systems so as to give additional value to aquaculture products, and ensure environmental protection, different approaches can be considered. First, improving diets could increase production and reduce the environmental impact of fish farming: the proportion of nutrients used by fish for growth can be maximized, thus reducing the amount of organic waste in the effluent, represented by the food component not treated, undigested, and not ingested by fish. The selection of highly digestible foods in quantities that do not exceed the assimilative capacity of fish, then, are two important factors to reduce potential environmental impacts. For young and adult fish, dry pellets represent major progress. Here, it is fundamental to determine the right dose of animal and vegetable proteins, fats, mineral salts, vitamins, and other additives, as well as the shape of the pellets and feeding frequency.

Moreover, partly due to the rapid expansion of aquaculture over the past 20 years (Naylor et al., 2000), the welfare of farmed fish has received increasing attention, not just for public perception, marketing, and production acceptance, but also often in terms of production efficiency, quality, and quantity (Broom, 1998; FSBI, 2002). One of the major themes of interest is that fish farming conditions provoke multiple stressing agents and many of these result in fish feeding behavior disorders (Ashley, 2007). Diet plays an important role in stress sensitivity. Fish are often

deprived of food before certain management procedures; this is designed to reduce physiological stress. For example, temporary starvation, prior to transport or treatment of disease serves to evacuate the fish's gut and to reduce metabolism, oxygen demand, and waste production. As fish are ectothermic, periods of food deprivation may be less detrimental than in endotherms (FSBI, 2002). It is not uncommon for some species in the wild to survive long periods of food deprivation (e.g., over winter), varying their ability to reduce metabolic costs when less food is available.

Generally, diet has an important influence on immuno-competence, how diseases develop after outbreak, and how fish respond to stressful aquaculture events. These factors have the potential to interact with food deprivation to affect welfare in either a positive or negative way. Consequently, there is economic and welfare-related pressure to ensure that both the frequency and the effects of a number of key management practices reduce stress responses in aquaculture species to a minimum (Ashley, 2007).

Continued research will further improve and benefit both aquaculture productivity and fish welfare (Ashley, 2007). Relatively few studies have investigated the effects of starvation on stress physiology and behavior, and the majority of work in this area concerns the effect of prolonged starvation on fish growth, muscle protein, and fat composition and also on changes in metabolic activity. Hence, the impact that food deprivation can have on the welfare of a fish may be relevant in relation to what is already known for vertebrates, under the same stress factor.

In 1908, Wilson and Osbourn described for the first time that a period of increased growth rate ensues a previously imposed growth restriction, with regard to beef steers. Subsequently, the mechanisms governing this faster rate of growth were studied by a number of researchers and the phenomenon termed "compensatory growth" (Bohman, 1955).

1.1 Compensatory growth in vertebrates

1.1.1 Characteristics of compensatory growth

In the natural environment, individuals of many species commonly experience periods of high food availability interspersed with periods of near or actual famine, as a consequence of temporal and spatial fluctuation in resources (Broekhuizen et al., 1994). To adaptat to a lifestyle involving alternating glut and scarcity, many organism exhibit faster growth during recovery from total or partial food deprivation than they do during periods of continuous food availability (Wilson and Osbourn, 1960). This phenomenon is called "compensatory growth" or "catch-up growth" and has been observed in invertebrates (Perrin et al., 1990; Bradley et al., 1991) as well as humans (Dulloo et al., 1997), mammals, and birds (Wilson and Osbourn, 1960; Hornick et al., 2000; Rincon and Leeson, 2002).

During compensatory growth, animals experiencing a period of growth depression may quickly achieve the same size-at-age as conspecifics experiencing environmental conditions that are more favorable. This accelerated growth might be controlled by feedback mechanisms, which adjust growth rates to restore the original growth trajectory (Tanner, 1963). Studies in vertebrates have shown that the magnitude of compensation was proportional to the nature, severity, and duration of undernutrition (Coleman and Evans, 1986). Moreover, the response varies largely and the expression of this growth regulation is dependent on several factors (Wilson and Osbourn, 1960; Ryan et al., 1993), including the stage of development at the start of the restriction period, the age at sexual maturity of individuals, and the pattern of re-alimentation (Ali et al., 2003).

Generally, the ratio between the size of compensating and control animals when the compensatory response decreased provides a measure of the effectiveness of the compensation. In cases of full compensation, the deprived animals ultimately attain the same body size as continuously fed contemporaries, whereas in partial compensation the deprived individuals fail to achieve the same size as the control group, also showing relatively rapid growth rates (Fig. 1.1). In some species, the compensation can be so intense that animals subjected to variable food supplies exhibit a higher net growth rate and a greater body size than those for whom food was continuously available (Fig. 1.1). This over-compensation seems to be a rare outcome (Jobling, 1994), however growth is better when the duration of growth

restriction is short and not too severe (Hornick et al., 2000). Finally, no growth compensation is observed when the re-alimented individuals resume growing at a rate characteristic of the size reached at the end of the food restriction period (Fig. 1.1). For example, very young animals that were severely feed restricted or affected by severe disease often fail to express compensatory growth (Coleman and Evans, 1986; Abdalla et al., 1988).

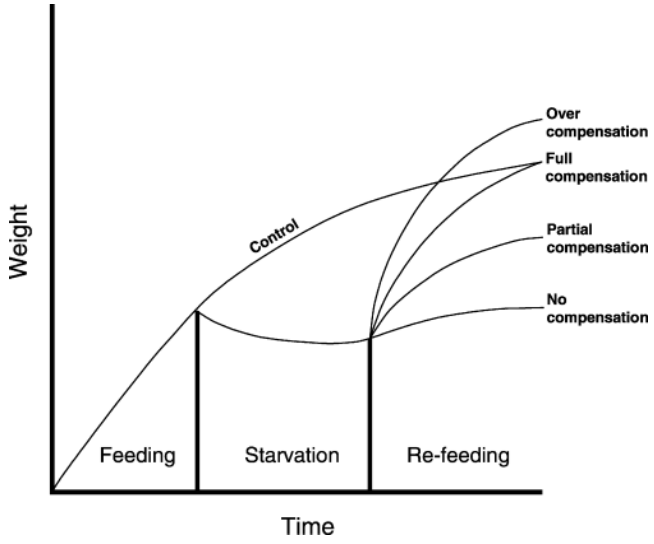

Figure 1.1 Idealized pattern of growth compensation based on Jobling (1994).

The main engine of compensatory growth is a relative increase in food ingestion, known as "hyperphagia" as a reaction to poor nutritional condition (Friedman, 1998). In order to increase its growth rate, an individual must either raise its resource intake or decrease its metabolic costs.

Large increases in ingestion rate under recovery conditions have been demonstrated in a variety of mammals (Weigle, 1994; Blum, 1997; Friedman, 1998). Gurney et al. (2003), using a series of mathematical energy-budget models, found that hyperphagia alone normally produces weak compensation and can never result in overcompensation. When combined with internal allocation, which routes a fixed fraction of net production to reserves, a strong compensatory response becomes the norm, and overcompensation occurs frequently.

Consequently, compensatory responses can be examined in relation to the growth of different tissues, organs, or primary body components.
The organism can experience a reduced growth of some body components without showing significant alteration in its size as a whole, due to a substantially different influence of undernutrition on growth dynamics (Broekhuizen et al., 1994).
The existence *per se* of the compensatory growth response is important to animal production: this phenomenon has clear ecological implications and has attracted considerable applied-research interest because of its potential impact on feed-lot husbandry (Hayward et al., 1997). Generally, studying how species locate and exploit food resources should be a basic issue from the behavioral and evolutionary perspective, while investigating in detail the effects of variable food intake on growth and growth efficiency in individuals could be essential for understanding the compensatory phenomenon.

1.1.2 Changes in body composition during compensatory growth

Several studies have assessed the reduced and consequent compensatory growth in mammals (Hornick et al., 2000), first of all focusing on the mechanisms underlying both these processes.
Growth results from the differences between tissue synthesis and degradation and losses of energy, nitrogen, and minerals from the body due to excretion.
During normal development, muscle shows initially the highest growth rate, followed by fat tissue. If growth rates are reduced, there is a sequentially and coordinated decrease in tissue turnover: the empty visceral fraction decreases, whereby fat deposits are affected more than protein deposits and the body becomes leaner (Hornick et al., 2000). Furthermore, some adipose compartments, such as subcutaneous fat, may be mobilized more easily than others (Yambayamba et al., 1996). When feed restriction is severe, weight losses are characterized by a sharp decrease in synthesis, indicating that these mechanisms are influenced far more by feeding intensity, than degradation processes. Generally, tissues are mobilized sequentially with an early consumption of a very labile protein compartment (Paquay et al., 1972) followed by mobilization of fat depending on the severity and duration of restriction whereas the protein pool is conserved insofar as

possible. Tissues are affected by growth restriction in relation to their metabolic activity (Drouillard et al., 1991); therefore, metabolically very active tissues or organs, such as liver or intestine, show the greatest weight losses.

Some studies in ruminants have reported that a moderate food restriction initially reduces basal metabolism, mainly caused by a decrease in the volume and the metabolic activity of the viscera (Ortigues and Durand, 1995; Yambayamba et al., 1996). Then, during the initial stage of compensation, mostly muscle and protein are deposited and cattle carcass composition, for example, is close to that of the restriction phase (Wright and Russel, 1991). Lastly, fat deposition takes over and the high body weight gains ultimately result from several processes, which include an enhanced efficiency of body growth and subsequent location performance.

In birds, for example broiler chickens, nutrient restriction is usually employed to tackle problems that accompany early-life fast growth rate such as increased body fat deposition, high incidence of metabolic disorders, and disease (Saleh et al., 2005; Teimouri et al., 2005). Khetani et al. (2009) demonstrated that broilers that have been deprived of feed for longer time periods were able to compensate growth and reach the market weight equally to those fed continuously. These findings comply with previous studies indicating that a period of slow growth for birds subjected to early feed restriction is usually followed by a period of rapid growth when refeeding, and the fast rate of body weight gain compensates for the delayed growth.

1.2 Compensatory growth in fish

The term "compensatory growth" was first used with regard to mammals and in a range of domesticated endotherms (Wilson and Osbourn, 1960); however only few studies had investigated this phenomenon in fish until the early 1990s when the subject attracted attention, especially in relation to aquaculture (Ali et al., 2003).

Teleosts are ectotherms with patterns of indeterminate growth; thus the compensatory process can be examined at almost every stage of their life cycles.

The responses of fish seem to be resemble those observed in mammals after a period of nutritional restriction: hyperphagia, rapid growth, and the repletion of energy reserves (Broekhuizen et al., 1994; Jobling, 1994).

Indeed, compensatory growth has been studied in more fish species than in any other vertebrate taxa, constituting the best source of information on this process in ectotherms (Ali et al., 2003). A disproportionate number of the available studies of compensatory growth have focused on six salmonid species; the number of studies in cyprinids is lower, but there are data on about 13 species (Russell and Wootton, 1992). A certain amount of information is present also for strictly marine species (Ali et al., 2003). However, there is evidence of interspecific variation in patterns of growth compensation; therefore, species in similar geographical ranges, with similar social behavior, and similar diets can present distinct compensatory capacities and different mechanisms of compensation (Sogard and Olla, 2002). This accentuates the need for a wider taxonomic coverage in the study of compensatory growth.

Compensatory performance has been studied in many fish species of economic and social importance for reasons of commercial exploitation, recreational fishing, or use in aquaculture. Thus, the obvious utility of research focusing on compensatory growth in fish has produced a series of studies on various commercial species (Broekhuizen et al., 1994).

1.2.1 Methodological studies and factors affecting compensatory growth

Little is known about the incidence, consequences, and importance of compensatory growth in natural populations (Ali et al., 2003). It is difficult to demonstrate compensatory response under natural conditions in manipulative field studies, such

as obtaining individuals of equivalent reproductive and development status, whose growth history was affected differentially by some identified or unknown environmental factors (Ali et al., 2003). For example, in the wild estimates of the size of individuals are required at different times and recapture rates of individually marked fish must be sufficiently high to generate adequate sample sizes. Indirect methods, such as back-calculation of size at known ages, can avoid costs and difficulties associated with tracking marked individuals (Nicieza and Braňa, 1993a,b), but have limitations since they only apply to some species and do not provide any information about the environmental factors influencing growth dynamics.

To assess the role that compensation can play as an important process of growth regulation in natural populations, the constraints in the habitat and ecology of the population that might evoke a compensatory response must first be defined.

Most of the empirical evidence of compensatory growth comes from manipulative experiments, and laboratory data on fish subjected to a reduction of food intake, mainly imposed with cyclic starvation and refeeding, provide the largest source of data (Broekhuizen et al., 1994).

Research on fish has also assessed the effects of growth depressors other than starvation, including rearing density, unseasonably low temperatures, exposure to hypoxia and reproductive effort. The literature contains mainly a number of careful measurements of fish growth trajectories under feeding regimens consisting of alternating periods of total or partial starvation followed by refeeding (Weatherley and Gill, 1981; Miglavs and Jobling, 1989a,b; Quinton and Blake, 1990). As in vertebrates, the initial phase of a bout of starvation shows a relatively rapid weight loss; then the rate of weight loss declines and remains at this lower value until death or reintroduction of food rations. During refeeding, starved fish initially gain weight at a rate similar to well-fed controls, but after a small amount of weight is gained, growth accelerates and remains high until most or all of the weight lost during starvation is regained (Fig. 1.2).

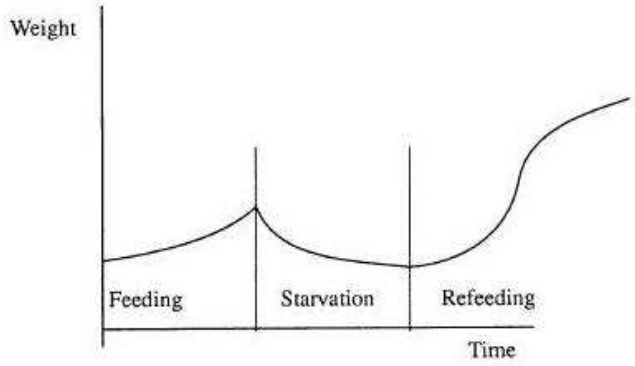

Figure 1.2 Typical pattern of weight change during feeding-starvation-refeeding cycle, in fish.

Clear evidence of the pattern of compensatory growth has emerged from experiments in which fish were exposed to a single period of total deprivation: full compensation was achieved by juvenile European minnow, *Phoxinus phoxinus* (Russell an Wootton, 1992; Zhu et al., 2001), and juvenile gibel carp, *Carassius auratus gibelio* (Xie et al., 2001) and was achieved or approached by three-spined stickleback, *Gasterosteus aculeatus* (Zhu et al., 2001). In these experiments, the deprived fish reached the mass of control fish after 2-4 weeks of refeeding and the trajectories of recovery were similar for both groups. There is also evidence that periods of food shortage combined with low temperature can provoke different patterns of compensation (Ali et al., 2003). In juvenile Atlantic salmon, *Salmo salar*, a 3-week period of unseasonably low temperature in early summer induced full compensation, but it was not displayed until the following autumn (Maclean and Metcalfe, 2001).

Compensation is shown to occur after a period of unseasonably low temperature with a reduced rate of food consumption even in sea bass (Pastoureaud, 1991), and in Atlantic cod, *Gadus morhua* (Purchase and Brown, 2001). By contrast, the lack of a compensatory growth response, for example in common carp, *Cyprinus carpio* (Schwarz et al., 1985) and in channel catfish, *Ictalurus puntatus* (Gaylord et al., 2001), may have reflected inadequate rates of feeding in the re-alimentation period, the effects of deprivations may not have been sufficient to trigger a

compensatory response, or the food restriction may even have exceeded a certain severity, as in higher vertebrates (Wilson and Osbourn, 1960; Ryan, 1990).
Density and social interactions between individuals also have consequences for compensatory growth: in juvenile Nile tilapia, *Oreochromis niloticus*, relative growth losses caused by crowding conditions were rapidly eliminated when the fish were returned to lower, control densities (Basiao et al., 1996).
However, comparisons between studies are generally difficult because of differences in experimental protocols, species, and ontogenetic stages.

1.2.2 Proximate causes of compensatory growth

Several factors could contribute to the compensatory growth observed during re-alimentation after a period of relative or total food deprivation or from other causes of growth depression. These include a rate of food consumption greater than that of continuously fed controls (hyperphagia), enhanced growth efficiency, reduced metabolic costs and reduced expenditure on locomotion.
Most experimental evidence suggests that growth operates by adjusting food intake in growth-depressed animals. Almost all studies measuring feeding rates have found that hyperphagia contributes significantly to the acceleration of growth, major if compared with the contribution of other variables.
Thus, hyperphagia is, by far, the main mechanism involved in the compensatory response (Ali et al., 2003).
Several studies have suggested that the hyperphagic response in fish was analogous to that observed in mammals and birds (Miglavs and Jobling, 1989a,b; Hayward et al., 1997: Ali and Wootton, 1998). The extent to which fasted fish display hyperphagia upon return to adequate feeding conditions may be related to the degree to which its energy reserves were depleted during fasting (Jobling and Miglavs, 1993). Moreover, the intensity of growth depression on the subsequent hyperphagia mainly affects the duration of the hyperphagic phase.
Despite the occurrence of this phenomenon, there is considerable interspecific variation in the temporal pattern of hyperphagia (Hayward et al., 1997; Ali et al., 2001; Wu et al., 2002), suggesting important interspecific differences in regulating

it. Dobson and Holmes (1984) have suggested that compensatory growth in fish could also be attributed to an increased efficiency of food utilization.

Studies in Arctic charr, *Salvelinus alpinus*, European minnows and the three-spined stickleback demonstrated improved food conversion efficiency, based on the relationship between mass of food consumed during re-alimentation and growth in body mass (Miglavs and Jobling, 1989a,b; Russell and Wootton, 1992; Jobling et al., 1994; Zhu et al., 2001). Furthermore, reduced activity during refeeding could contribute to compensatory gain by increasing the proportion of the energy available to growth. Wieser et al. (1992) suggested that whole-body metabolic rate of fish decreased in response to deprivation: individuals showed lower routine rates of respiration associated with a reduction in locomotion and decline in the activity of some major glycolytic and glycogenolytic enzymes in swimming muscles.

Understanding how standard metabolic rates react during a phase of compensatory growth could be relevant as this would provide useful information about the costs and risks associated with rapid growth.

1.2.3 Modelling compensatory growth

Numerous models of fish growth have been reported in the literature (Wootton, 1998), complex bioenergetic models which partition the total metabolic expenditure into many terms, corresponding to expenditures associated with locomotion, tissue synthesis, and tissue maintenance (Kitchell et al., 1977; From and Rasmussen, 1984). Most models, including those that describe energy flow into and out of the fish in detail, treat all internally stored energy as interchangeable with the momentary state of the fish.

Broekhuizen et al. (1994) proposed a simple, physiologically based model in which fish tissues are divided into reserve and structural components. Reserves correspond broadly to lipid stores and parts of the musculature that can be remobilized; structural tissues include skeletal, circulatory, and nervous tissues. The first component is the part of the total weight that can be mobilized to meet maintenance costs during periods of starvation, while the second does not decrease. The model proposes that fish attempt to maintain an optimum ration of

reserve for structural tissues and regulate feed intake and metabolism based on the difference between the achieved and optimum ratio.

A related model is the lipostat of Jobling and Johansen (1999). This model suggests that the ratio of fat and lean body mass is an indicator of fish nutritional status and that appetite is regulated in relation to lipid levels. This model shows that hyperphagia and the consequent compensatory response cease as lipid levels reach the optimal levels.

Clear evidence compatible with these two models was obtained in salmonids (Broekhuizen et al., 1994; Jobling and Johansen, 1999; Johansen et al., 2002).

1.2.4 Proximate fish composition during food deprivation and recovery

Considering the detailed body component distribution may offer clues as to the changes in proximate composition of a fish's body and to the control of appetite during deprivation and refeeding.

Fish metabolism is largely based on lipids and proteins (Jobling, 1994), with lipids stored in liver, viscera, and muscle. During starvation, lipids are broken down early as shown for the eel, *Anguilla anguilla* (Larsson and Lewander, 1973), sea bass (Stirling, 1976), North pike, *Exos lucius* (Ince and Thorpe, 1976), rainbow trout, *Oncorhynchus mykiss* (Jezierska et al., 1982) and Nile tilapia (Satoh et al., 1984).

Lipid levels change both before and during compensatory growth. For example, starvation in Arctic charr resulted in significant decreases in liver and viscera as proportions of body mass and in the lipid energy:protein energy ratio; restoration of satiation feeding was followed by significant increases in percentages of lipid in liver and viscera, compared to continuously fed controls, with whole-body ratios also rising (Miglavs and Jobling, 1989a).

A comparison between temporal changes in food intake and body composition suggested that the level of food intake in re-alimented fish decreased as visceral fat contents approached the level of controls fed a satiation level (Ali et al., 2003).

Protein levels are also influenced by starvation: muscle RNA concentrations reflect different somatic growth rates induced by different levels of feedings because of the role RNA plays in protein synthesis (Buckley, 1979; Westerman and Holt, 1988). In juvenile Atlantic salmon, RNA concentrations were significantly lower in the starved

group than in control fish (Arndt et al., 1996). A study in carp showed that starved individuals had lower white muscle RNA and liver RNA concentration than fed fish, indicating reduced protein synthesis under food restriction (Bastrop et al., 1991).

Carbohydrates play only a minor role as storage materials in fish. Particularly, the role of glucose in regulating appetite in teleosts is unclear and, in comparison to mammals, fish do not regulate plasma glucose well (Mommsen, 1998). Carter et al. (2001) suggest that this makes it unlikely that glucose plays an important role in the regulation of food intake in fish.

Finally, starvation results in tissue hydration (Jobling, 1980; Miglavs and Jobling, 1989a). An increase in tissue hydration plays a role in the limitation of the loss or even the maintenance of wet body mass during starvation (Love, 1970). Whether the level of tissue hydration plays any direct role in regulating appetite in teleosts is not known. It is also unclear which, if any, of the changes observed during deprivation provide the error signals for compensatory growth when food becomes freely available, but surely there is an underlying hormonal regulation.

1.3 Endocrine regulation of fish growth

In vertebrates, growth is under genetic control and is also affected by nutrient availability and a multitude of environmental factors (Moriyana et al., 2000) to which the brain responds with appropriate modifications through hormonally mediated pathways (Duan, 1998). A central step in the endocrine pathway is the growth hormone (GH)-insulin-like growth factors (GH-IGFs) axis (Fig. 1.3).

Growth hormone is produced by the pituitary gland under the control of the hypothalamic hormones; it binds to its receptors in the target organs mainly in the liver and stimulates synthesis and release of insulin-like growth factor-I (IGF-I) (Moriyana et al., 2000). This is precisely why the majority of the growth-promoting effects of growth hormone are believed to be mediated by IGF-I (Duan, 1998; Moriyana et al., 2000), which in turn is structurally related to IGF-II (Shamblott and Chen, 1992). Both these substances, termed "insulin-like" because of their ability to stimulate glucose uptake into muscle and fat cells (Randle, 1954), are well known for promoting many anabolic responses in a variety of target cells in both endocrine and autocrine/paracrine fashions (Jones and Clemmons, 1995). IGF-I is involved in the regulation of protein, lipid, carbohydrate, and mineral metabolism in the cells, cell differentiation and proliferation, and ultimately body growth (Moriyama et al., 2000).

In mammals and birds, the increase in the number of muscle fibers (hyperplasia) stops at, or shortly after birth (Campion, 1984) and muscle growth is mainly caused by outgrowth of existing fibers (hypertrophy). In contrast, fish muscle continues to grow and the addition of new fibers continues to represent an important contribution to normal muscle growth well into adulthood (Weatherley et al., 1988). Thus, this suggests that the IGF system plays a special role in muscle maintained at higher levels for much longer throughout adult life in fish.

In salmonids, salmon GH administration increases plasma IGF-I and hepatic IGF-I mRNA levels in a dose-dependent manner (Moriyama et al., 1994). Moreover, it was seen that GH administration excites an increased growth that might be attributed to increased feeding and food assimilation, thus suggesting that GH acts directly on the central nervous system to modulate feeding behavior (Johnson and Bjornsson, 1994; Silverstein et al., 2000). However, the role of GH on the control of feeding is still controversial.

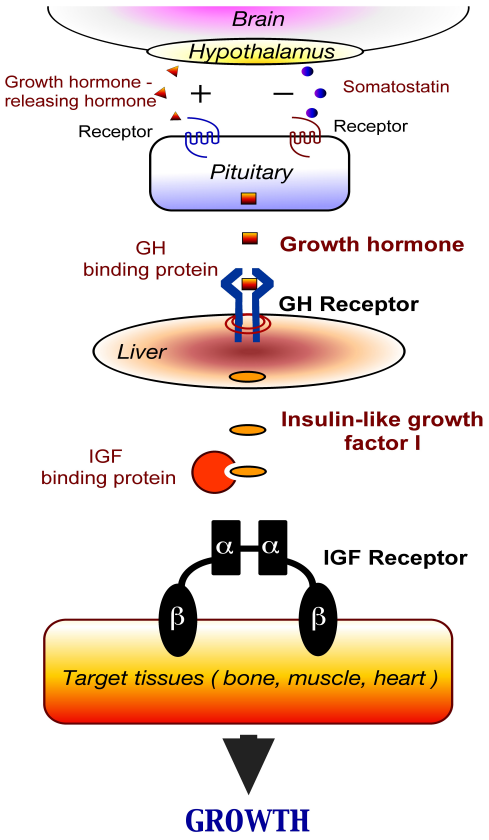

Figure 1.3 General scheme of the endocrine regulation of growth. Transmembrane receptor mediates the action of hypothalamic hormone, growth hormone, and insulin-like growth factor-I (IGF-I). Growth hormone and IGF-I circulate in blood tightly bound to specific binding proteins (Moriyama et al., 2000).

Fish IGF-I cDNA was first cloned from the liver in coho salmon, *Oncorhynchus kisutch* (Cao et al., 1989) and IGF-II cDNA in rainbow trout (Shamblott and Chen, 1992). Since then, the nucleotide sequence of IGF-I and IGF-II cDNAs has been determined in a number of teleosts including catfish (McRory and Sherwood, 1994); gilthead seabream (Duguay et al., 1996); common carp (Liang et al., 1996); goldfish, *Carassius auratus* (Kermouni et al., 1998); tilapia, *Oreochromis mossambicus* (Reinecke et al., 1997); Japanese eel (Yamaguchi et al., 1999), and sea bass (Terova et al., 2007).

IGF-I and IGF-II transcripts have been detected in many fish tissues, liver contained the greatest amount, which is in keeping with liver being the major site of their production (Duan, 1998).

In various fish species, both hypertrophic and hyperplasic components of muscle growth are stopped during fasting, indicating that the cellular aspects of muscle growth are influenced by feeding status of the fishes (Kiessling et al., 1990). Accordingly, the hepatic and muscular IGFs mRNA levels should depend on individual feeding status.

Subsequently, Duan and Plisetskaya (1993) demonstrated that food restriction decreases the IGF-I mRNA levels in liver in coho salmon, whereas refeeding of the starved fish increased in hepatic IGF-I expression.

In rainbow trout, muscular IGF-I and IGF-II mRNA levels have also been found to be lower in starved fish than in refed fish (Chauvigné et al., 2003).

Terova et al. (2007) studied IGF-I and IGF-II expression levels in liver and muscle of experimental fish and confirmed that both genes were involved in the compensatory growth induced by refeeding. Fasting of sea bass induced a significant decrease in the mRNA copy number of IGF-I and IGF-II in both liver and muscle, indicating that both systemic and local IGFs levels were affected. A dramatic increase in the amounts of IGF-I transcripts in liver and muscle was observed during the recovery from starvation; the IGF-II levels were also up-regulated in both tissues but more gradually. These differences in the level of IGFs may imply different means of regulating of gene expression and different roles in the growth of sea bass.

All these results indicate that nutritional status has a profound effect on the GH-IGF-I axis in fish, and fish muscle growth is considered to be an integrated process which depends for the most part on nutrient availability (Navarro and Gutiérrez, 1995).

In this context, a number of factors influencing the activation and proliferation of myogenic precursor cells have been identified in higher vertebrates. These factors, besides the IGFs, include members of the fibroblast growth factor (FGF) family, especially FGF6 (Olwin et al., 1994), and myostatin, a member of the transforming growth factor-β superfamily (McPherron et al., 1997).

FGFs are potent, positive regulators of muscle growth and FGF6 may play a specific role since its expression profile is essentially restricted to developing adult skeletal muscle (Pizette et al., 1996). The prolonged fish hyperplasia is associated with a continuous expression of FGF6 up to the adult stage and it is involved both in

proliferation and differentiation of the myogenic lineage by stimulating myoblast proliferation (Rescan, 1998). In contrast, myostatin (MSTN) is a negative factor and inhibits myoblast proliferation. MSTN seems to act predominantly on muscle growth. It is the main factor involved in depressing both the number of myoblasts that reach terminal differentiation divisions and, to some extent, the degree of fiber enlargement (Terova et al., 2006). The biological significance of these factors was most strikingly demonstrated by genetic ablation using homologous recombination techniques in mice: FGF6 has been shown to enhance satellite cells proliferation in vitro (Kästner et al., 2000) and null mutation of MSTN gene resulted in animals with more muscle mass and higher numbers of fibers (McPherron et al., 1997).

Since the initial characterization of MSTN and FGF6 in mice, dozens of additional cDNAs have been isolated in different vertebrate species, whereas orthologues from commercially important fish species have only been identified in a few species. Furthermore, only little is known about the involvement of these genes in modulating muscle growth in response to different feeding regimens.

Previous studies in rainbow trout (Chauvigné et al., 2003; Montserrat et al., 2007) did not detect changes in FGF6 expression during compensatory growth induced by refeeding. As with FGF6, myostatin mRNA levels were found to be unchanged in muscle from fasted and refed animals. These observations led to the conclusion that change in myostatin transcription likely does not mediate muscle recovery elicited by refeeding in trout (Chauvigné et al., 2003; Montserrat et al., 2007).

By contrast, Terova et al. (2006) quantified MSTN abundance levels and demonstrated that MSTN is involved in the compensatory growth of sea bass: the increase in MSTN mRNA levels in the muscle of fasted fish indicate that this autocrine/paracrine growth factor has a role in modulating muscle growth in response to different feeding regimens. Up-regulation of MSTN during fasting and its decrease during refeeding are in line with the notion that myostatin has a negative influence on muscle hypertrophy and hyperplasia. Unlike MSTN, muscular FGF6 mRNA levels were not significantly affected in fed, fasted or refed sea bass (Terova et al., 2006), but these results cannot exclude that FGF6 activity in fasting muscle may be affected by other mechanisms such as post-translational processing, secretion or interaction with proteins.

1.4 Control of appetite in fish

In all vertebrates, appetite regulation is a complex phenomenon involving elaborate interactions between the brain and peripheral signals. The brain, particularly the hypothalamus, produces key factors that either stimulate (orexigenic) or inhibit (anorexigenic) food intake (Volkoff et al., 2005).

In mammals, distinctive neural systems are associated with peptides that regulate appetite for fat, protein, and carbohydrates (Hoebel, 1997) and several studies have shown that the hypothalamus is mainly involved in this control of food intake (Demski and Northcutt, 1983).

In fish, appetite is probably under comparable multifactorial control (Fletcher, 1984; DePedro and Bjornsson, 2001) and a number of peptides homologous to the mammalian appetite-regulating peptides have been isolated, although the details probably differ in some respects from endotherms.

In his lipostatic model, Kennedy (1953) already hypothesized that humoral signals generated in proportion to adipose mass provide input (feedback) to the areas of the brain that control feeding and energy expenditure. Thus, after a period of food restriction, the negative feedback signals that inhibit feeding are reduced because of fat loss. The result is that elevated food intake is maintained until the levels of fat and, hence, the negative feedback signals return to normal. Evidence that one such negative feedback signal was a circulating "satiety" factor came from a parabiosis study in rats (Hervey, 1959). Subsequent experiments concluded that the regulation of both food intake and body energy reserves in the form of fat were probably mediated by a blood-borne factor that acted at the level of the hypothalamus. In mammals, the brain neuropeptide "leptin or *obese/ob* protein" has been extensively explored and demonstrated to be a central link between adiposity, appetite and energy homeostasis in several species (Hoebel, 1997; Friedman and Halaas, 1998; Altmann and Von Borell, 2007; Spady et al., 2008). Leptin is a 16 kDa protein encoded by the ob gene that is produced and secreted mainly by adipocytes, but also by certain tissues, including brain and gastric epithelium (Harvey and Ashford, 2003). It affects food intake by inhibiting hypothalamic orexigenic pathways and stimulating anorexigenic pathways.

Leptin shows both short- and long-term effects. Short-term postprandial increase in plasma leptin levels inhibits food intake, whereas long-term energy status is

communicated to the brain based on the daily mean plasma leptin levels (Crespi and Denver, 2006). In teleosts, leptin was first identified in pufferfish, *Takifugu rubripes* (Kurokawa et al., 2005), and subsequently in other species, including common carp (Huising et al., 2006); Arctic charr (Frøiland et al., 2009); rainbow trout (Kling et al., 2009); Japanese medaka, *Oryzias latipes* (Kurokawa and Murashita, 2009); Atlantic salmon (Rønnestad et al., 2010), and grass carp, *Ctenopharyngodon idella* (Li et al., 2010).

In Atlantic salmon, Rønnestad et al. (2010) detected higher leptin expression in tissue typically associated with lipid storage, including belly flap, white muscle, and visceral adipose tissue, and also in the gastrointestinal tract, in accordance with results in mammals. They also examined the long-term effects of rationed feeding in fish, which resulted in a significantly lower growth and lower leptin mRNA expression in the main lipid-storing tissues. This finding suggests a possible link between gene expression and energy status of Atlantic salmon, in line with the mammalian model, even if no differences were observed in plasma levels. In contrast, plasma leptin levels increased during 3-week fasting of rainbow trout (Kling et al., 2009), indicating potential differences in responses of the leptin system to long-term and short-term changes in nutritional status in fish. At present, data on leptin expression and function in teleosts are still too scarce to make any generalizations regarding its role in regulating food intake, growth, and adiposity (Rønnestad et al., 2010).

Another key element of this communication process is the hunger-inducing hormone ghrelin, which is believed to convey information about nutrient availability from stomach to the brain (van der Lely et al., 2004). The characterization of ghrelin has made an important contribution to our understanding of hypothalamic control of food intake.

Ghrelin, a 28-amino acid peptide (Kojima et al., 1999) synthesized in the stomach and brain, is involved in the control of energy homeostasis and increases food intake in mammals (Wren et al., 2001; Horvath et al., 2003).

Among the teleosts, ghrelin has been identified in several species: goldfish (Unniappan et al., 2002); Japanese eel, *Anguilla japonica* (Kaiya et al., 2003a); Nile tilapia (Perhar et al., 2003); rainbow trout (Kaiya et al., 2003c); channel catfish, (Kaiya et al., 2005); black seabream, *Acanthopagrus schlegeli* (Yeung et al., 2006),

and sea bass (Terova et al., 2008). As in mammals, ghrelin mRNA is highly expressed in fish stomach, and moderate levels are detected in the brain (Kaiya et al., 2003a,b; Unniappan et al., 2002).

In goldfish, for example, there is a postprandial decrease in ghrelin mRNA expression in the hypothalamus and gut, whereas periprandial expression changes in the same tissues, providing further support to the notion that this peptide has orexigenic effects. Finally, 7 days of starvation induced increases in ghrelin mRNA expression in the goldfish hypotalamus and gut (Unniappan et al., 2004a,b).

Fasting-induced increases in ghrelin levels were confirmed by Terova et al. (2008) in a study that supports a possible role for ghrelin in regulation of food intake in sea bass. Their results demonstrate that this peptide is abundantly expressed in the stomach of this species; in particular, fasting for 5 weeks significantly increased the amount of ghrelin transcript, whereas during refeeding there was a significant increase in mRNA transcript levels as compared to control animals until the 4th day of recovery. Subsequently, 10 days after refeeding ghrelin mRNA abundance levels decreased compared to the previous time point tested, remaining still higher than the control values, and then returned to original levels after 21 days of refeeding.

This study was the first to be conducted over such a long fasting period and it demonstrated up-regulation of ghrelin upon fasting and down-regulation during refeeding, results that are in line with previous interpretations of ghrelin functioning as an orexigenic gastrointestinal peptide, even in fish. Furthermore, as proof of compensation responses, fasted sea bass showed hyperphagia, a simple but compelling example of food intake regulation triggered during refeeding, as a mechanism by which fish compensated for their growth loss.

Generally, the differences in ghrelin responses to fasting may reflect differences in energy metabolism and the extent to which fasted fish display high levels of ghrelin expression may be related to the degree to which their energy reserves were depleted during fasting (Terova el al., 2008).

1.5 Fish nutritive physiology during compensatory growth

Growth and efficiency of food use depend on the physiological and biochemical capacities of fish to digest and transform ingested nutrients. However, many biotic and abiotic factors can strongly influence the processes related to digestion, uptake, and transformation of nutrients (Furné et al., 2008). Studies of digestive enzyme activity in fish have helped to define the limits of dietary protein (Twining et al., 1983), lipids and carbohydrates (Spannhof and Plantikow, 1983), recommending different proportions of dietary macronutrients for the best nutrient utilization. Understanding how the digestive machinery functions also helps determine how nutrients are best digested (Glass et al., 1989; Kolkovski, 2001). In short, studies on the activity of digestive enzymes in fish can elucidate some aspects of fish nutritive physiology and contribute to resolving nutritional situations (Hidalgo et al., 1999), such as the lack of food.

Fish farms routinely submit fish to starvation situations during a given period in certain situations. Accordingly, investigating the digestive machinery under these conditions could indicate to what point starvation governs the utilization of nutrients after refeeding. Moreover, by studying enzymes during refeeding the response capacity of fish and the degree of reversibility of these alterations can be evaluated (Furné et al., 2008).

Because diet can affect digestive enzyme activities (German et al., 2010), several studies have investigated how the nutritional status of fish affects these enzymes. For example, Bélanger et al. (2002), studying Atlantic salmon submitted to prolonged starvation, observed a decline in enzymatic activities in the intestine and in the pyloric ceca, which recuperate after refeeding; their results also suggest that faster compensatory growth would be supported by enhanced digestive capacity.

The utilization of energy reserves during starvation periods appears to differ according to fish species. Thus, some fish use protein as the main energy source, while others use lipids (Alliot et al., 1974), and the enzymatic profile is related to being a carnivorous, omnivorous, or herbivorous species.

Furné et al. (2008) showed that protease and amylase activity is higher in sturgeon, *Acipenser naccarii*, than in trout, in accordance with its greater capacity to digest carbohydrates, categorizing the sturgeon's habits as being closer to those of an omnivore. This study also revealed that after a long starvation period, the

machinery for digesting macronutrients was altered in both these species. Previously, Terova et al. (2007) already demonstrated that the nutritional state of sea bass influences the levels of expression of progastricsin (or pepsinogen C), the inactive precursor of the proteolytic enzyme gastricsin responsible for the initial and partial hydrolysis of the dietary proteins.

In fish, the amino acid sequences of pepsinogen have been reported in several species; however only few studies have examined the gene expression of such enzyme (Douglas et al., 1999; Murray et al., 2006). Terova et al. (2007) reported the impact of chronic food deprivation on the copy number of progastricsin mRNA in stomach of sea bass: upon fasting progastricsin mRNA levels were down-regulated and up-regulated during refeeding. These results are in line with previous interpretations of gastricsin function and provide further support for its proteolytic actions. Therefore, during fasting the stomach is nearly deplete of gastric juices, while the presence of food during refeeding produces abundant secretion.

Sea bass individuals showed a remarkable increase in transcriptional levels of progastricsin mRNA during short-term refeeding (4 days), which might be explained by the great quantity of protein taken with the food in the first days of refeeding. In fact, refeeding of sea bass after a long starvation period was also marked by hyperphagia as early as the first day, as a compensation response.

Since that time, more emphasis has always been placed on understanding and clarifying how digestive enzyme expression levels are related to the feeding status of fish, mainly fish farmed species.

1.6 Dietary lipid requirements and effect of nutritional status on fish

The nutrients required by fish for growth, reproduction, and other normal physiological functions do not vary greatly among species, but differences in fatty acid requirements represent notable exceptions (Lovell, 1991).

Fatty acids are the most important component common to all classes of lipids and are widely represented in living organisms, in which they have structural, metabolic, and energetic functions.

Fatty acids are classified according to the length of the carbon chain (short, medium, or long), the number of double bonds (unsaturated, mono-, or polyunsaturated), and, most importantly, the essential they are in the diet (essential or nonessential).

Saturated fatty acids may be of dietary or endogenous origin and the more abundant ones in animal lipids are palmitic (16:0) and stearic acid (18:0).

Non vertebrate species possess desaturase enzymes that can insert double bonds over the nine-tenth carbon atom fatty acids, so that the polyunsaturated fatty acids (PUFA) produced by the organism all have double bonds in the carboxyl portion of the molecule, a feature rendering them to be considered nonessential. Otherwise, essential fatty acids are generally divided into two classes: omega 3 (ω-3) and omega 6 (ω-6), depending on the location of their first double bond in the methyl portion of the molecule. The precursors of the two groups are α-linolenic acid (18:3 n-3) and linoleic acid (18:2 n-6), both of plant origin.

European aquaculture, particularly of marine fish, is dependent upon feed grade fisheries to provide fish meal and oils traditionally used as the main protein and lipid sources in the feeds. The general trend to increase lipid content in aquafeeds for marine fish to improve growth, feed conversion and protein utilization has produced an increase in the demand for fish oil in aquaculture. However, the capacity of the fisheries to cope with the increasing demand for fish oil has reached the limit of sustainability, owing to problems such as overfishing, climate alterations and increasing demand from other sectors (Sargent et al., 2002). Thus, alternative oils are needed to supply lipids for aquafeeds, and those from plant seeds such as linseed, rapeseed, or soybean oils are good candidates. Among the teleosts, freshwater species differ from marine species as regard their fatty acid requirements. The former have a recognized capacity to desaturate and elongate

the fatty acids with 18 carbon atoms, linoleic (18:2 n-6) and linolenic (18:3 n-3) acids, into highly unsaturated fatty acids (HUFA) with 10 and 22 carbon atoms such as arachidonic (ARA, 20:4 n-6), eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic (DHA, 22:6 n-3) acids (Sargent et al. 2002) (Fig. 1.4). The HUFA play a major role in eicosanoid production (AA and EPA), vision (DHA), brain development (DHA), and regulation of expression of several genes involved in lipid metabolism (Forman et al., 1997).

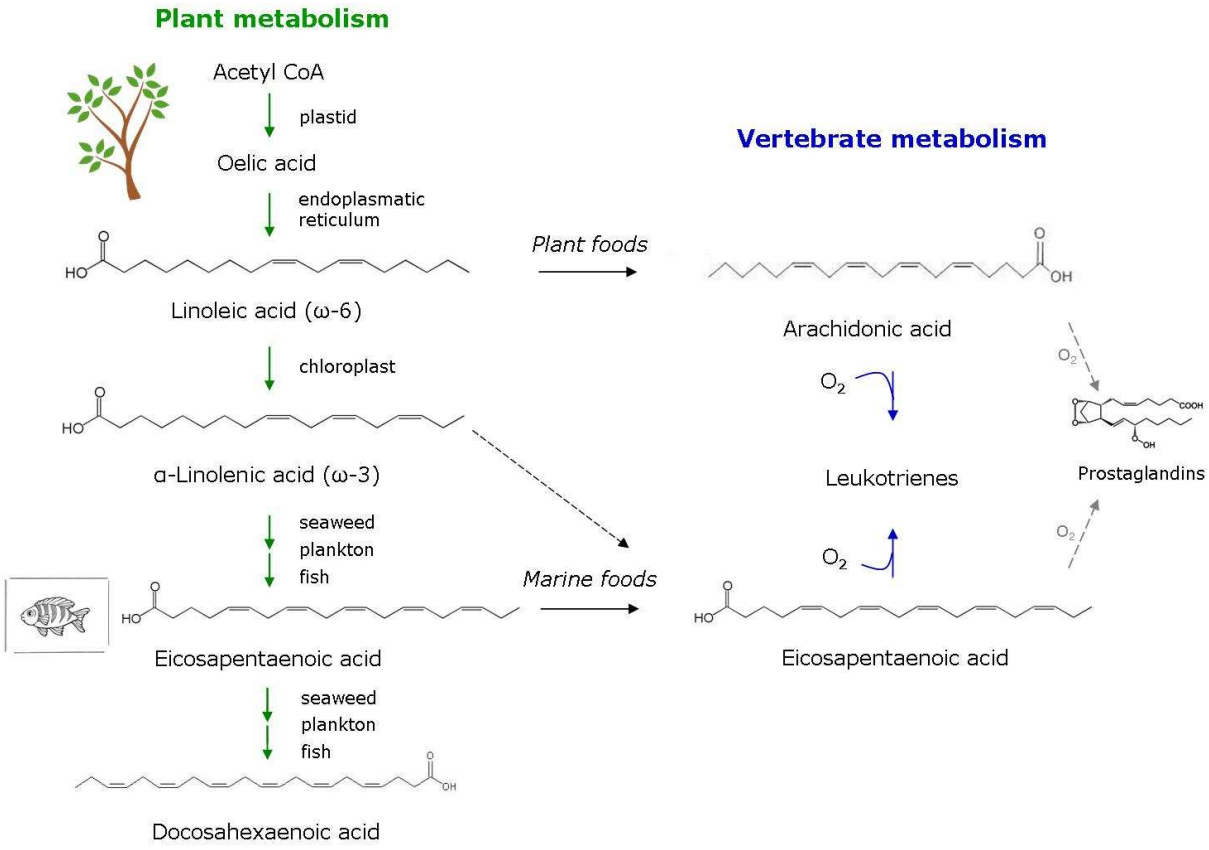

Figure 1.4 Origin and metabolism of fatty acids -6 and -3 in plants and vertebrate.

In contrast to freshwater fish, marine species are assumed to have a deficient capacity to bioconvert 18C precursors into HUFA and, hence, require preformed HUFA in their diet. Although long recognized, the reasons for this deficiency in marine fish remain unclear. On the one hand, it could be due to a gene loss corresponding to an adaptation to the n-3 HUFA-rich marine food web, as also

suggested for terrestrial carnivores (Sargent et al., 1995). On the other hand, marine fish show a deficiency in desaturase enzymes, probably due to repressed desaturase activity associated with high levels of HUFA usually present in the diet (Olsen et al., 1990), as previously seen in mammals.

By clearly determining the essential fatty acid requirements in marine fish and understanding the molecular basis of HUFA biosynthesis and how it is regulated in fish, the use of vegetable oils can be manipulated and optimized in aquaculture.

Replacing fish oil with vegetable oils in marine fish diets has been well studied: not only can fish health be compromised by including certain vegetable oils, but the nutritional quality of the flesh can also be affected by modifying muscle fatty acid profiles, which includes a reducing n-3 HUFA, and particularly EPA (Izquierdo et al., 2003) and altering the relationship between n-3 and n-6 fatty acids. Moreover, vegetable oils can affect the flesh quality of aquacultured fish in terms of n-3 HUFA source for human consumption. To avoid these problems, we need to know how the different vegetable oil diets influence fish fatty acid biosynthesis.

1.6.1 Genes involved in fatty acid metabolism: Δ6 desaturase, lipin and PPAR

In recent years, significant progress has been made in characterizing fatty acid desaturases involved in HUFA synthesis. Δ6 fatty acid desaturase has been shown to be the enzyme responsible for the first and rate-limiting step in the biosynthesis of HUFA from C_{18} PUFA (Fig. 1.5), and it has been isolated from several fish species, including zebrafish, *Danio rerio* (Hastings et al., 2001); common carp (accession no. AF309557); gilthead seabream (Seiliez et al., 2003); Atlantic cod (accession no. DQ054840); rainbow trout (accession no. NM_001124287), and Atlantic salmon (Zheng et al., 2009).

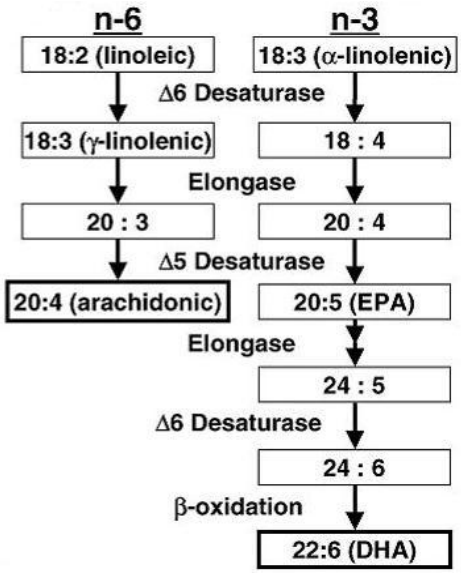

Figure 1.5 The actual pathways for *n-6* and *n-3* HUFA biosynthesis.

Many reports have described the cloning, tissue distribution, and the nutritional regulation of Δ6 desaturase in fish. Referring to the latter, a study in rainbow trout demonstrated for the first time that different levels of Δ6 desaturase transcripts were present and that the enzyme was nutritionally modulated in fish fed diets with varying sources of dietary lipids (Seiliez et al., 2001). Inhibition of desaturase gene expression by dietary HUFA is shown also in gilthead seabream (Seiliez et al., 2003): Δ6 desaturase transcripts appear to be depressed in fish fed a HUFA-rich diet compared to fish fed a HUFA-free diet, indicating a nutritional regulation similar to that reported for HUFA on mammalian desaturases (Cho et al., 1999a,b). These findings are confirmed by the study of Izquierdo et al. (2008), showing that very high contents of linseed oil in gilthead seabream diet inhibited desaturase activity, while diets formulated with soybean or rapeseed oils increased the relative expression of this enzyme.

In contrast to these studies, there is no evidence of an effect of starvation and subsequently refeeding on the Δ6 desaturase mRNA levels in fish. Likewise, an abundance of information is available about genes involved in lipid biosynthesis but no one has demonstrated any trend during compensatory growth.

Biochemical and genetic studies have defined many of the key players in triacylglycerol (TAG) biosynthesis, storage, and lipolysis in adipose tissue. Among these, the lipin protein family appears to have important dual cellular roles, serving as an enzyme required for TAG and phospholipid biosynthesis, and as a transcriptional coactivator in regulating lipid metabolism genes (Fig. 1.6; Reue and Zhang, 2008). In mammals, three members of the lipin gene family encode three lipin protein isoforms: lipin-1 is expressed at highest levels in adipose tissue, and skeletal muscle and can also be detected in liver, brain, kidney, and other cell types; lipin-2 is most prominently expressed in liver and brain, whereas lipin-3 is present at low levels in several tissues, notably small intestine and liver (Donkor et al., 2007).

The three mammalian lipin genes differ in their tissue expression patterns, suggesting that they have unique physiological roles. At present, little is known about the physiological role of the two family members lipin-2 and lipin-3, while lipin-1 appear to play important roles in metabolic homeostasis in both adipose tissue and skeletal muscle. Consistent with its metabolic functions in adipogenesis, high levels of lipin-1 in transgenic mice promoted obesity, while lipin deficiency prevented normal adipose tissue development, with dramatically reduced tissue mass and aberrant adipocyte gene expression (Phan et al., 2004).

Lipin gene orthologs are well conserved from unicellular eukaryotes to mammals, suggesting a fundamental biological role. In teleosts, lipin genes have only been studied in zebrafish, showing the presence of predicted lipin-1 and lipin-2 members. Generally, fish seem to have two lipin-related genes and pathways equivalent to those involved in the biosynthesis and catabolism of fatty acids in mammals, but it remains to be determined whether the same molecular mechanisms control these pathways (Boukouvala et al., 2004).

In higher vertebrates, peroxisome proliferator-activated receptors (PPAR) have emerged as central factors in the control of the expression of genes involved in lipid homeostasis. Three PPAR isotypes, termed α, β/δ, and γ, have been identified in different organisms, including mammals, birds, and amphibians.

PPARs activate the transcription of target genes by binding specific ligands including a natural fatty acids, especially polyunsaturated fatty acids, eicosanoids, and arachidonate derivates, and many coactivators, such as the lipin protein (Fig. 1.6).

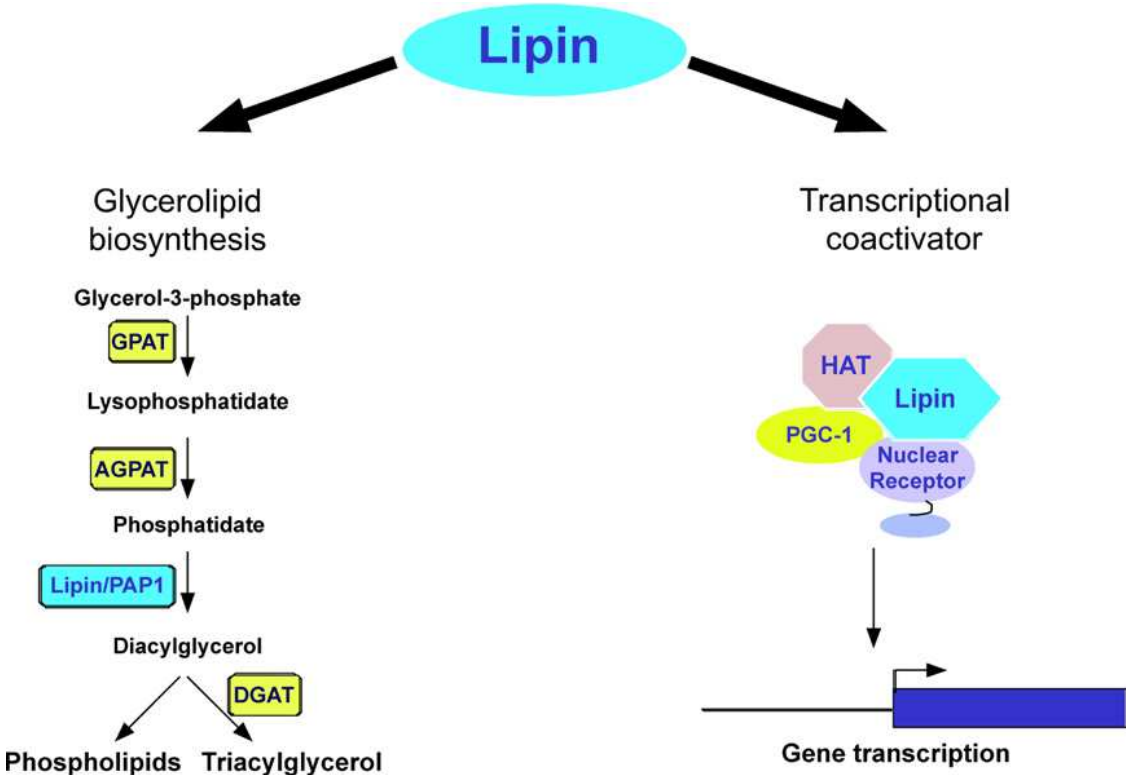

Figure 1.6 Dual cellular functions of lipin proteins. Lipin-1, -2 and -3 all exhibit phosphatidate phosphatase activity, which plays a role in triglyceride and phospholipid biosynthesis. Lipin-1 has also been shown to act as a transcriptional coactivator in liver, directly interacting with nuclear receptors such as PPAR.

In contrast to the large amount of information available on mammalian PPARs, reports for PPARs in fish are mainly limited to the identification of PPARγ in some species: sea bass (Boukouvala et al., 2004); gilthead seabream (Leaver et al., 2005); Japanese sea bass, *Lateolabrax japonicas* (accession no. DQ345545); common dentex, *Dentex dentex* (accession no. EF470299); plaice, *Pleuronectes platessa* (accession no. AJ243956), and other species. The presence of even a single PPAR isotype in lower vertebrate raises intriguing questions regarding the evolution of the structure and function of these receptors. However, the exact number of genes and the presence of distinct PPAR isotypes in fish have not been determined. In addition, it is not known whether the different PPAR isotypes, if present in fish, act through similar mechanisms and perform the same critical functions in lipid metabolism as they do in mammals. Thus, the study of piscine

PPARs could provide information about manipulating fatty acids metabolism in fish to develop interventions targeted specifically at these key regulators factors.

Boukouvala et al. (2004) have reported that sea bass PPARs are structural homologs of the mammalian PPARα, β/δ, and γ isotypes, also showing a very similar tissue expression profile. Thus, PPARα is mainly expressed in the liver, PPARγ in adipose tissue and PPARβ in all tissue tested with its highest levels in the liver, where it is also the dominant isotype expressed. Recently, Leaver et al. (2005) published an interesting study carried out on two species of marine fish, plaice and gilthead seabream, in which cDNAs and genes encoding three PPAR isotypes are reported for the first time. Although there are differences in the genomic organization of the fish PPAR genes, sequence alignments and phylogenetic comparisons showed that the fish genes are homologs of mammalian PPAR counterparts. Furthermore, they found that nutritional status strongly influenced the expression of all three PPAR isotypes in seabream: the mRNA expression levels of PPARα, β, and γ were increased in the livers of fasted fish and decreased following refeeding, but no PPAR isotypes expression in intestine or adipose tissue was affected by nutritional status.

These findings are in contrast to those in both mice and rats, in which fasting was shown to provoke a down-regulation of PPARs and a substantial decrease in PPARγ in adipose tissue (Escher et al., 2001).

Closer consideration of the structure of the piscine PPARs ligand binding domains might explain these differences. In fact, amino acid residues critical for ligand binding in mammalian PPARs are not conserved in fish and it may be that fish species have used PPARs in different ways than terrestrial vertebrates do (Leaver et al., 2005).

1.7 Protein and amino acid requirements and effect of nutritional status on fish

Fish require both proteins and lipids for growth, reproduction, and other normal physiological functions . Proteins are the major organic material in fish tissue, making up about 65-75% of the total on a dry-weight basis. Fish consume protein to obtain amino acids, which are absorbed from the intestine tract and continually used by various tissue to synthesize new protein; inadequate protein content in the diet results in a reduction in growth and loss of weight. When excess protein is supplied in the diet, only part is used for protein synthesis, and the remainder is converted into energy (Wilson and Halver, 1986).

Optimal dietary protein concentrations for fish are dictated by a delicate balance between the dietary protein-to-energy ratio, plus protein quality (amino acid balance), and non-protein energy sources (i.e., amount of fat in relation to carbohydrates) (Millikin, 1982). Fish do not have a true protein requirement, but require a well-balanced mixture of nonessential and essential amino acids: fish require the same 10 essential amino acids as warm-blooded animals (Wilson and Halver, 1986). One of the striking differences in nutrition between fish and farm animals, however, is that fish require far less energy for protein synthesis.

Fish exert relatively less energy to maintain position and to move in water than do mammals and birds on land, and they excrete most of their nitrogenous wastes as ammonia instead of urea or uric acid, thus losing less energy in protein catabolism and excretion of nitrogenous waste (Lovell, 1991).

Significant interspecies differences with regard to protein requirements appear to exist in fish. These variations can be attributed to a number of factors such as differences in basal diet composition, size and age of fish, genetic difference, and feeding rate (Fournier et al., 2002).

Given the increase in aquaculture production and the implications of poor protein utilization on nitrogenous losses in effluents, there is an increasing need to optimize the supply of protein and nonessential amino acids. Thus, studies have focused on understand the underlying mechanism of protein digestion and absorption in fish.

Digested dietary proteins in both teleosts and mammals are subject to hydrolysis by a range of proteases and peptidases that generate a mixture of free amino acids and small peptides which are absorbed across the apical membranes of enterocytes. In particular, the intraluminal products of protein digestion are

predominantly di- and tripeptides, not amino acids, as was widely believed over 30 years ago (Adibi, 2003).

Cellular transport of amino acids and small peptides (di- and tripeptides) is thus a key final step in assimilation by the intestine. Furthermore, intestinal peptide transports is of major nutritional significance because many amino acids are more rapidly and efficiently absorbed in peptide form (Gilbert et al., 2008a).

1.7.1 The oligopeptide transporter 1, PepT1

Responsible for the uptake of dietary di- and tripeptides in cells is the oligopeptide transporter 1 (PepT1), an integral plasma membrane protein. It transports peptides against a concentration gradient by coupling the movement of substrate across the membrane with the movement of protons down an inwardly directed electrochemical proton gradient (Fig. 1.7). PepT1 presents a unique feature, i.e., the capability of sequence-independent transport of nearly all possible di- and tri-peptides, including differently charged species (Daniel et al., 2006).

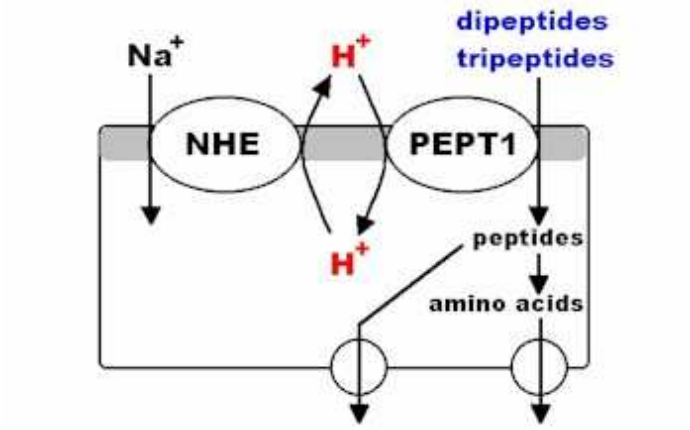

Figure 1.7 PepT1 transports oligopeptides by using a inwardly directed H^+ gradient across the brush-border membrane.

Since the initial characterization of the PepT1 transporter by expression cloning in rabbit in 1994, dozens of additional cDNAs have been isolated in different vertebrate species. It has been shown to be prominently expressed in epithelial cells of small intestine and, in addition to the large number of di- and tripeptides, some peptidomimetics also serve as substrates for PepT1 including beta-lactam

antibiotics, selected angiotensin-converting enzyme inhibitors, some antiviral nucleoside prodrugs, and omega-amino fatty acids. The amino acid sequence predicts 12 transmembrane domains with both amino and carboxy termini facing the cytoplasmic side and a large loop between the membrane spanning helices 9 - 10 (Fig. 1.8). In contrast, among teleosts PepT1 has only been cloned and characterized in a few species, such as zebrafish (Verri et al. 2003); Atlantic cod (Rønnestad et al., 2007b); Asian weatherloach, *Misgurnus anguillicaudatus* (Goncalves et al., 2007); rainbow trout (Ostaszewska et al., 2009); and China rockfish, *Sebastes nebulosus* (accession no. EU160494). Results from some studies in fish demonstrated that PepT1 is mainly expressed at the intestinal level, although moderate levels have also been detected in a variety of other tissues (Verri et al., 2003; Rønnestad et al., 2007b). Moreover, accumulating evidence indicates that PepT1 may have a major role in fish nutrition (Dabrowski et al., 2005), transporting large amounts of amino acids in peptide form and providing essential nutrients for fish growth and the required energy for metabolism and reproduction.

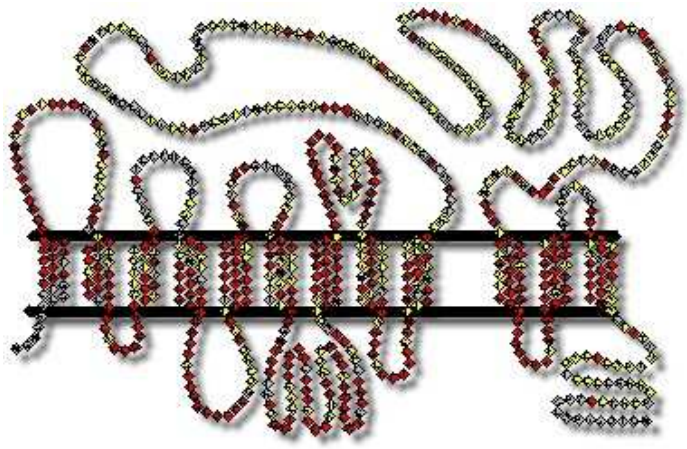

Figure 1.8 The PepT1 amino acid sequence predicts 12 transmembrane domains with both amino and carboxyl termini facing the cytoplasmic side and a large loop between the membrane spanning helices (MSH) 9 and 10.

In recent years, the influence of dietary protein on PepT1 expression and activity has been an area of active research. Results from these studies demonstrated that expression levels and function of PepT1 are very responsive to dietary treatments. In general, peptide transporter expression and activity increase in conjunction with dietary protein and peptide levels, suggesting that up-regulation via a high-protein diet may represent a mechanism for taking advantage of the abundant resource, whereas up-regulation shown in response to a lack of substrate appears to be a compensatory mechanism to scavenge amino acids in the lumen. Moreover, the magnitude of the response in changes of PepT1 expression and activity probably depends on the duration of a particular dietary manipulation, availability of substrate, and amino acid composition (concentrations of free and peptide-bound) (Gilbert et al., 2008). The corresponding information for fish is completely unknown; however, this would be of great importance, in particular for the farmed species raised in feed-based aquaculture systems. In fact, from the commercial perspective, it is very costly to use fish dietary protein as a nutrient and even fractional improvements in this area have the potential to save the aquafeed industry millions of euros and also to reduce the amount of nitrogen excreted into the environment.

1.8 Project purpose

Many aquaculture management tools currently in use have conservation value. They are designed to maintain stocks of commercially important species above target levels. However, their limitations are evident from continuing declines in fish stocks throughout the world.

Conservation concerns have been secondary to economic imperatives, and marine conservation efforts have seriously lagged behind those on land. However, there is now an urgent need to ramp up protection of the marine environment both to recover fisheries and safeguard biodiversity. There is great enthusiasm for the new aquaculture practices because a growing body of theoretical and empirical work suggests they can simultaneously meet conservation and fishery objectives. In addition to recovering stocks of target species, other key fishery management benefits claimed for marine reserves include the development of natural age structures of exploited species, protection of genetic variability, and improving fish welfare.

In accordance with all this knowledge, ever-increasing numbers of studies focus on defining and measuring the effects of aquaculture procedures on welfare to produce data and recommendations for best practice, which are then applicable in the natural environment.

The aim of the present study is to identify molecular markers whose expression is affected by a stress factor that can also be found under natural conditions, where analysis is more difficult. A possible cause of poor fish welfare considered here is dietary manipulation, which includes temporary starvation and subsequent refeeding.

As elucidated in the introduction, a period of fasting and refeeding in fish induced a compensatory response, a phenomenon that can be well applied in particular for the farmed species raised in feed-based aquaculture and also for the commercial perspective of the aquafeed industry.

The study will be conducted in sea bass, the commercially most important marine species for Mediterranean aquaculture. The experiment involves cessation of food intake and subsequent recovery of food intake in sea bass identifying genes whose expression is affected by the dietary manipulation and that can thus be used as

molecular markers, and understanding their contribution to the compensatory growth induced by fasting and refeeding in sea bass.

In particular, we will study the activity and the molecular basis of regulation of some genes capable of responding to dietary stimuli, including genes involved in the control of lipid and protein metabolism.

To our knowledge, there is no information regarding the response of such genes at the mRNA level to different feeding regimens in fish. Indeed, much is known about the physiological and biochemical response of fish to starvation, but only little information is available at the molecular level. In particular, we know nothing about the response of all the genes potentially involved in starvation and refeeding, although this knowledge could provide an accurate estimate of gastrointestinal function in fish and is relevant to understanding the mechanism by which their expression is regulated.

In this regard, the research focuses on identifying three genes involved in fatty acids metabolism: Δ6 desaturase, lipin, and PPARγ. First, the target genes cDNA encoding sequences were isolated in sea bass and then we assessed the impact of chronic feed deprivation and subsequent refeeding on mRNA abundance levels in liver and proximal intestine of this teleost, with the aim to relate these expression levels to feeding status.

Subsequently, the same analysis will be performed for PepT1 mRNA expression levels in the intestine of sea bass, with the purpose of verifying the existence of a possible relationship between the expression and activity of this oligopeptide transporter and the nutritional status of fish. Moreover, we will assess the spatial distribution of mRNAs PepT1 in all sea bass tissues to determine whether it has a major role in fish nutrition.

2. Materials and methods

2.1 Animals and feeding protocol

One month prior to starting the experiment, 140 sea bass were randomly stocked into four tanks of 2 m^3 each, with 35 fish per tank, and allowed to acclimate. During the 1-month acclimation period, all fish were fed Hendrix-Skretting® Power Excel feed for marine fish. The tanks were connected to a sea water recirculation system. Other water conditions were: temperature 20 ± 2°C, pH 7, and total ammonia < 0.2 mg/L; dissolved oxygen was maintained over 99 % of the saturation by insufflating pure O_2 to the system.

At the start of the experiment, all the fish were weighed, and two of the tanks were randomly assigned to each of two treatments. Fish in these two tanks were fed to apparent satiety (fed control), whereas fish in the other two tanks were deprived of food for 35 days and then refed to apparent satiety for 21 days with the same type of feed utilized before fasting. Feed consumption (g) in each tank was estimated from the difference between feed delivered into the tank and uneaten feed which was collected from the bottom of the tank. Feed intake was converted to grams of feed consumed per kg body weight (BW) of the fish per day. At the beginning of the experiment the mean body weight of the sea bass was 119.34 ± 15.27 g for the control group and 118.41 ± 18.37 g for the "food-deprived" group. Five fish from each of the experimental groups were sampled at the following time points: before fasting (day 0), 4 days after fasting, at the end of fasting (35 days), and then sequentially at 4, 14, and 21 days following refeeding. Fish were sampled 15 min before the scheduled feeding time. For the molecular biology analysis stomach, proximal intestine and liver were isolated, frozen immediately in liquid N_2, and stored at -80 °C. For the spatial distribution analysis of PepT1, the following parts of the digestive tract were dissected out: gastroesophageal junction, stomach fundus, pyloric antrum, pyloric caeca, 10 subsequent segments of the intestine dissected at every centimetre, and rectum (Fig. 2.1). In addition, other tissues such as liver, gill, heart, kidney, ovary, brain, muscle and spleen were isolated, frozen immediately in liquid N_2, and stored at -80 °C.

All the fish in the tanks were weighed also at the end of fasting period and after 21 days of refeeding. They were rapidly anesthetized with tricainemethane sulfonate

(MS-222, 100 mg/l) and body weight and length (standard + total) were measured and used to calculate the condition factor: (k= body weight x 100/ standard body length^3).

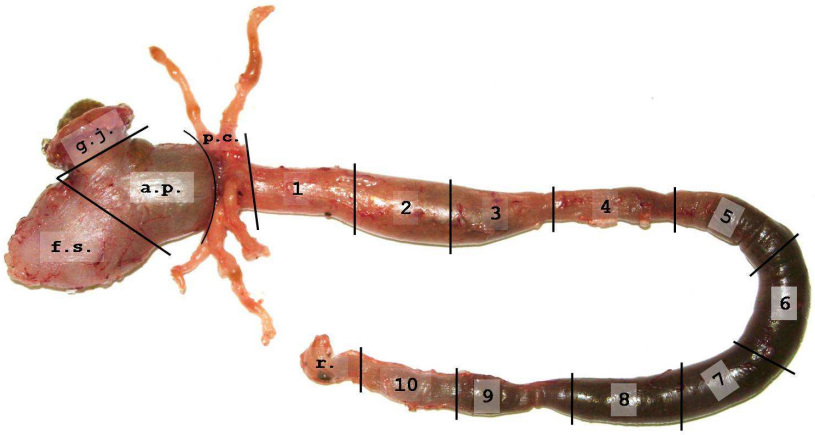

Figure 2.1 Picture of sea bass digestive tract: gastroesophageal junction (g.j.), fundus stomach (f.s.), pyloric antrum (p.a.), pyloric caeca (p.c.), ten adjacent segments starting after the pyloric area (1-10), and rectum (r).

2.2 Preparation of total RNA and cDNA synthesis

Total RNA was extracted from sea bass stomach, proximal intestine, liver and all other tissues isolated using PureYield RNA Midiprep System (Promega, Italy), following the manufacturer's protocol. The quantity of the RNA was calculated using the absorbance at 260 nm. The integrity and relative quantity of RNA was checked by electrophoresis. After extraction, 3 µg of total RNA was reverse transcribed into cDNA in a volume of 12 µl containing 1 µl of oligo dT16 primer (50 pmol) and 1 µl of 10 mM dNTPs. This mix was heated at 65 °C for 15 min and chilled on ice, and then 4 µl of 5X reverse transcription buffer, 2 µl of 0.1 M DTT, 1 µl RNaseOUT, and 200 units of Moloney murine Leukemia virus reverse transcriptase were added to a final volume of 20 µl, as described in the M-MLV Reverse Transcriptase kit (Invitrogen). After incubation at 37°C for 50 min, the reaction was stopped heating at 75 °C for 15 min.

2.3 Cloning and sequencing

To perform PCR, an aliquot of 4 µl of the resulting cDNA from each tissue was amplified with 1.5 U GoTaq Polymerase (Promega) in 50 µl of final volume containing 10 µl buffer, dNTPs 10 mM, and 50 pmol of each target gene designed RT-PCR primer set (Fig. 2.2, 2.3 and 2.4; Table 2.1).

A total of 30 cycles (10 touchdown) of the PCR amplification were performed for all primer sets, using an automated Thermal Cycler (Mycycler, Biorad). The annealing temperatures depended on the melting temperatures of the primer set used. An aliquot of each sample was then electrophoresed on 1 % agarose gel in 1X TAE buffer (Eppendorf) and bands were detected by ethidium bromide staining. The positive control consisted in a master mix of cDNA and cytoplasmatic β-actin primers (accession no. AY148350) (Terova et al., 2005), while the negative control consisted of total RNA added to the RT reaction mixture without reverse transcriptase and subsequently amplified using the same set of primers and the same conditions. The negative control confirmed the absence of genomic contamination.

The PCR products from Δ6 desaturase, lipin and PepT1 primers amplifications were cloned using the pGEM®-T Easy cloning vector system (Promega, Italy) and subsequently sequenced in both directions (T7 and SP6).

2.4 5' and 3' RACE analysis

The 5' ends of the cDNA sequences were analyzed using a commercial kit for rapid amplification of the cDNA ends (5' RACE) system (Invitrogen). First-strand cDNA was synthesized from poly $(A)^+$ RNA using SuperScript™ II reverse transcriptase and gene-specific antisense primer 5'_GSP1 (Fig. 2.2, 2.3 and 2.4). A poly dCTP tail was added to the single-stranded cDNA present using terminal deoxynucleotidyl transferase (Invitrogen). Second-strand cDNA synthesis was carried out using TaqPolymerase (Qiagen), a poly d(G) anchor primer (RACE_AAP), dNTP mix, and buffer. The reaction was incubated in a thermal cycler at the following conditions: 40 °C for 5 min, 72 °C for 2 min, and then the temperature was increased at 80 °C. At this point, a nested sequence-specific primer 5'_GSP2 (Fig. 2.2, 2.3 and 2.4) and a nested anchor primer RACE_AUAP were added for amplification at the following

conditions: 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min (30 cycles), last extension time 72 °C for 10 min; then kept at 4 °C. A quantity of 1 µl of a 1:10 dilution of the PCR products was re-amplified using the nested anchor primer RACE_AUAP and the nested sequence-specific primer 5'_GSP3 (Fig. 2.2, 2.3 and 2.4). The resulting products were run on a 2% agarose gel, purified, cloned into the pGEM-T vector, and sequenced.

The 3'-end tails of the cDNAs were analyzed using the 3'RACE System for Rapid Amplification of cDNA Ends (Invitrogen). First-strand cDNA was synthesized from poly $(A)^+$ RNA using SuperScript™ II reverse transcriptase and Adapter Primer (AP). The target cDNA was amplified using the cDNA synthesis reaction, TaqPolymerase, dNTP mix, and buffer. The reaction was incubated in a thermal cycler at 80°C for 1.5 min. At this point, a nested sequence-specific primer 3'_GSP1 (Fig. 2.2, 2.3 and 2.4) and a nested anchor primer RACE_AUAP were added for the amplification. Of a 1:10 dilution of the PCR products 1 µl was re-amplified using the nested anchor primer RACE_AUAP and the nested sequence-specific primer 3'_GSP2 (Fig. 2.2, 2.3 and 2.4). The resulting products were run on a 2% agarose gel, purified, cloned into the pGEM-T easy vector, and sequenced.

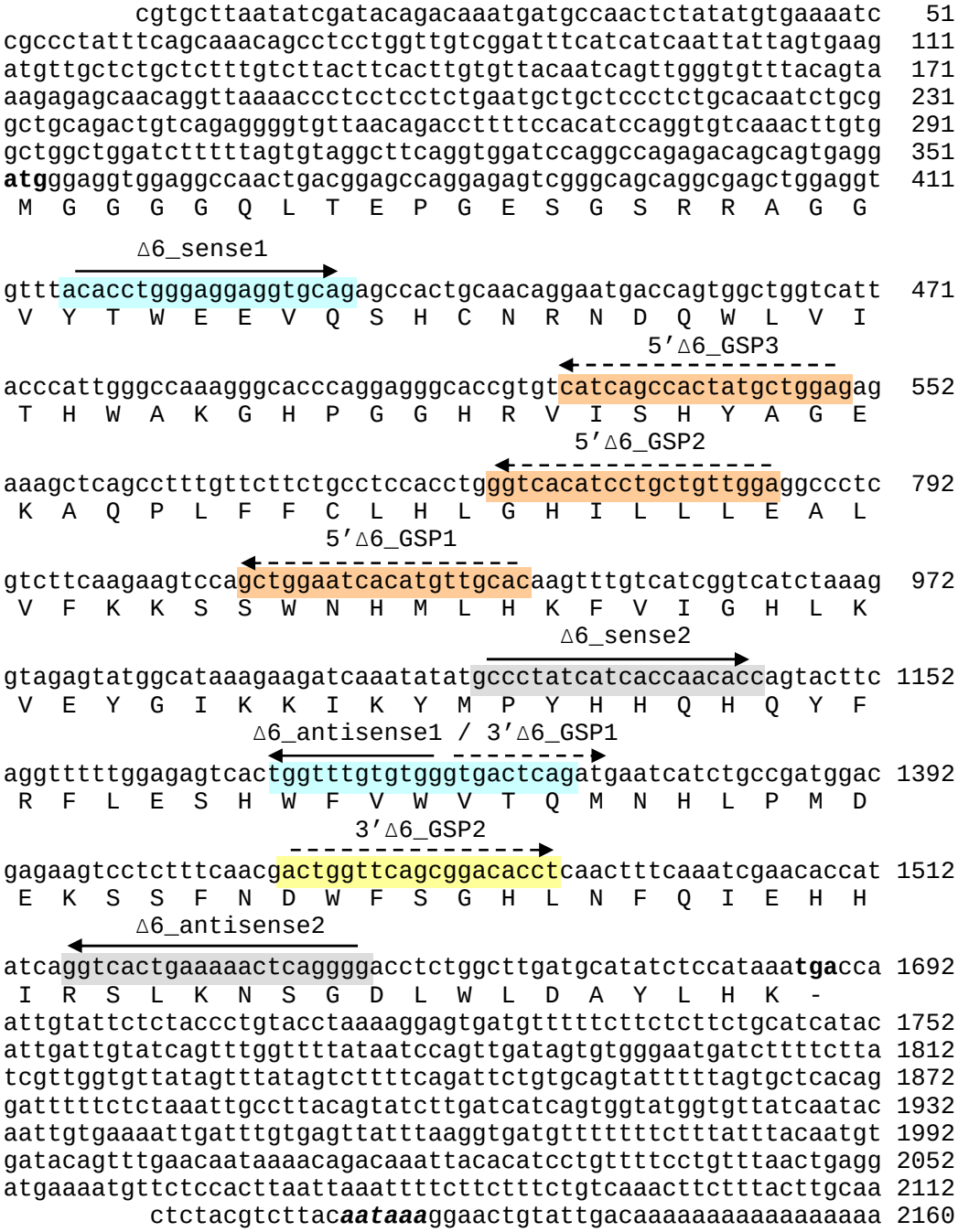

Figure 2.2 The nucleotide sequence of sea bass Δ6 desaturase (accession no. EU647692), with deduced amino acids shown below the sequence in single-letter code. Nucleotides are numbered to the left. The locations of the primers used in PCR of the full-length transcript and in the 5′ or 3′ RACE are also indicated by solid and broken horizontal arrows, respectively.

Figure 2.3 The nucleotide sequence of sea bass lipin (accession no. EU644089), with the deduced amino acids shows below in single-letter code. Nucleotides are numbered to the left. The locations of the primers used in PCR of the partial cDNA sequence are indicated by solid horizontal arrows.

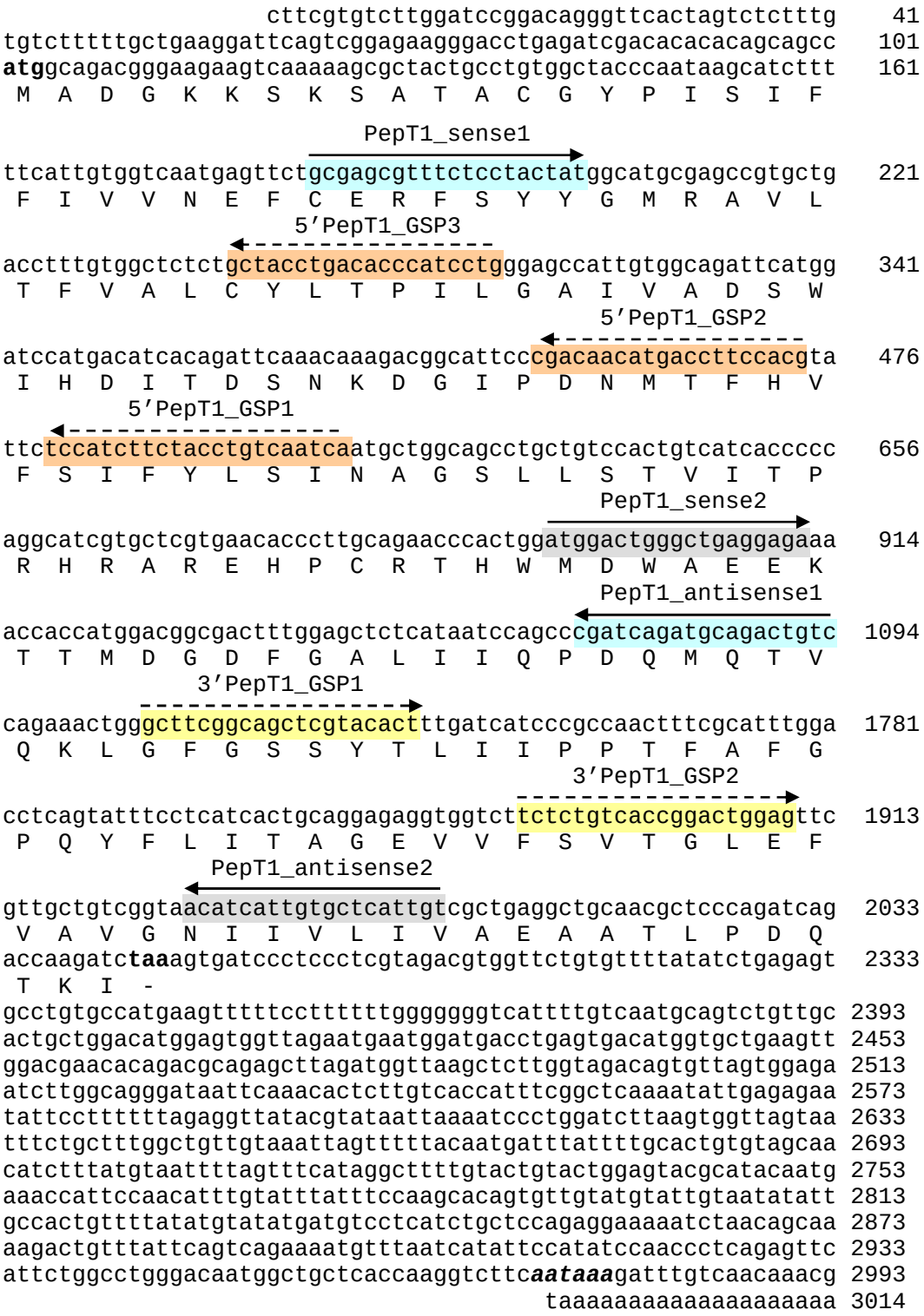

Figure 2.4 The nucleotide sequence of sea bass PepT1 (accession no. FJ237043), with deduced amino acids shown below the sequence in single-letter code. Nucleotides are numbered to the left. The locations of the primers used in PCR of the full-length transcript and in the 5′ or 3′ RACE are also indicated by solid and broken horizontal arrows, respectively.

2.5 Quantitative real-time RT-PCR

2.5.1 Generation of in vitro-transcribed cRNAs for standard curves

The number of each target gene transcript copies could be absolutely quantified by comparing them with a standard graph constructed using the known copy number of mRNAs of each gene. For this, a forward and a reverse primer were designed based on the mRNA sequences of *sea bass* Δ6 desaturase (accession no. EU647692), lipin (accession no. EU644089), PPARγ (accession no. AY590303), and PepT1 (accession no. FJ237043). These primer pairs were used to create templates for the *in vitro* transcription of cRNAs for each gene. The forward primers were engineered to contain a T7 or a T3 phage polymerase promoter gene sequence to their 5' end and used together with the reverse specific primer in a conventional RT-PCR of total sea bass proximal intestine (for Δ6 desaturase and PepT1) and liver (for lipin and PPARγ) RNAs. RT-PCR products were then checked on a 2.5% agarose gel stained with ethidium bromide, cloned using pGEM®-T Easy cloning vector system (Promega, Italy) and subsequently sequenced in the SP6 direction.

In vitro transcription was performed using T7 or T3 RNA polymerase and other reagents supplied in the Promega RiboProbe *In Vitro* Transcription System kit according to the manufacturer's protocol.

The molecular weight (MW) of the *in vitro*-transcribed RNAs were calculated according to the following formula:

MW = [(*no of A bases*) x 329.2) + (*no of U bases*) x 306.2) + (*no of C bases*) x 305.2) + (*no of G bases*) x 345.2)] + 159.

Spectrophotometry at 260 nm gave a concentration of each cRNA. Therefore, the concentration of the final working solutions were calculated, and expressed as n° of molecules/µl.

The results were:

- Δ6 desaturase MW of 124,129.6 and $2.47x10^{12}$ molecules/µl;
- lipin MW of 129,743.4 and $1.62x10^{12}$ molecules/µl;
- PPARγ MW of 97,703.2 and $3.43x10^{12}$ molecules/µl;
- PepT1 MW of 114,920.4 and $2.88x10^{12}$ molecules/µl.

Table 2.1 Primers used

Primer	Sequence 5′ – 3′	Purpose
PepT1-sense1	GCGAGCGTTTCTCCTACTAT	RT-PCR
PepT1-antisense1	GACAGTCTGCATCTGATCG	RT-PCR
PepT1-sense2	ATGGACTGGGCTGAGGAGA	RT-PCR
PepT1-antisense2	ACAATGAGCACAATGATGT	RT-PCR
Lipin-sense1	TACCACAAAGTGAGCCAGA	RT-PCR
Lipin-antisense1	TGGTCTTTGCGTGCTCCT	RT-PCR/ standard curve
Δ6-sense1	ACACCTGGGAGGAGGTGCAG	RT-PCR
Δ6-antisense1	CTGAGTCACCCACACAAACCA	RT-PCR
Δ6-sense2	GCCCTATCATCACCAACACC	RT-PCR
Δ6-antisense2	CCCCTGACTTCTTCAGTGACC	RT-PCR
PPARγ-sense1	CACTCCCTTGACATGAAGCATT	RT-PCR
PPARγ-antisense1	GTATCGTCCTGGTGCTTGGA	RT-PCR/ standard curve
5′ Δ6_GSP1	AGAGCCAGATGATGAGCCAG	5′ RACE
5′ Δ6_GSP2	TCCAACAGCAGGATGTGACC	5′ RACE
5′ Δ6_GSP3	CTCCAGCATAGTGGCTGATG	5′ RACE
3′ Δ6_GSP1	TGGTTTGTGTGGGTGACTCAG	3′ RACE
3′ Δ6_GSP2	ACTGGTTCAGCGGACACCT	3′ RACE
5′PepT1_GSP1	TGATTGACAGGTAGAAGATGGA	5′RACE
5′PepT1_GSP2	CGTGGAAGGTCATGTTGTCG	5′ RACE
5′PepT1_GSP3	CAGGATGGGTGTCAGGTAGC	5′ RACE
3'PepT1_GSP1	GCTTCGGCAGCTCGTACACT	3'RACE
3'PepT1_GSP2	TCTCTGTCACCGGACTGGAG	3'RACE
PepT1_T7promoter	GTAATACGACTCACTATAGGGGGAATGTGGCAT TCACACC	standard curve
PepT1_antisense3	GTCCATCTTGAGCCCTGCT	standard curve
PPARγ_T3promoter	CAATTAACCCTCACTAAAGGGACACTCCCTTGA CATGAAGCAT	standard curve
Δ6_T3promoter	CAATTAACCCTCACTAAAGGGAATCTTCAGTAA GGACCCAGAT	standard curve
Δ6_antisense3	GATGTCCATCGGCAGATGATT	standard curve
Lipin_T3promoter	CAATTAACCCTCACTAAAGGGATGTACCACAAA GTGAGCCAG	standard curve

2.5.2 Generation of standard curves

The cRNAs produced by *in vitro* transcription were used as quantitative standards in the analysis of experimental samples. Defined amounts of cRNAs at 10-fold dilutions were subjected in triplicates to real-time PCR using one-step TaqMan EZ RT-PCR Core Reagents (Applied Biosystems, Italy), including 1x Taqman buffer, 3 mM MnOAc, 0.3 mM dNTP except dTTP, 0.6 mM dUTP, 0.3 µM forward primer, 0.3 µM reverse primer, 0.2 µM FAM-6 (6-carboxyfluorescein-labeled probe), 5 units *rTH* DNA polymerase, and 0.5 units AmpErase UNG enzyme in a 25 µl reaction. AmpErase® uracil-N-glycosylase (UNG) is a 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-N-glycosylase gene. UNG acts on single-and double-stranded dU-containing DNA. It acts by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA. For Taqman® assays, AmpErase® UNG treatment can prevent the reamplification of carry over PCR products from previous PCR reactions. When dUTP replaces dTTP in PCR amplification, AmpErase UNG treatment can remove up to 200,000 copies of amplicon per 50 µl reaction. RT- PCR conditions were: 2 min at 50°C , 30 min at 60°C, and 5 min at 95°C, followed by 40 cycles consisting of 20 s at 92°C, 1 min at 62°C. The Ct values obtained by amplification were used to create standard curves for target genes.

2.5.3 Quantitation of transcripts by one-step RT-PCR TaqMan system

A hundred nanograms of total RNA extracted from the experimental samples were subjected, in parallel to triplicates of 10-fold-diluted, defined amounts of standard cRNAs, to real-time PCR under the same experimental conditions as for the establishment of the standard curves. Real-time Assays-by-Design^{SM} PCR primers and gene-specific fluorogenic probes were designed by Applied Biosystems (ABI). Primer sequences and Taqman® probes of the obtained target genes were the followings:

Target gene: Δ6 desaturase

Forward primer: 5′- ATGATTTCCCGCCGTGACT -3′

Reverse primer: 5′- ACAGGTAGCGAAGGTAGTAAGACAT -3′

Taqman® probe: 5′- ACCAAGCCAGATCCAC -3′

Target gene: lipin

Forward primer: 5′- GGCCCAGTGCTGCTGA -3′

Reverse primer: 5′- CCTCCCTGTGCAAAGCAGAAA -3′

Taqman® probe: 5′- CCCCAGCAGCCTTT -3′

Target gene: PPARγ

Forward primer: 5′- TGCTGGTGTGGCCTATGAC -3′

Reverse primer: 5′- ATGTTGGTGTAGTCCATGTTGGT -3′

Taqman® probe: 5′- CAGAGCGAAGAACACC -3′

Target gene: PepT1

Forward primer: 5′– GCTACCCTCTGGCCTTTGG –3′

Reverse primer: 5′- GAACACAATCAGAGCTACCACCAT -3′

Taqman® probe: 5′- TCCCCGCTGCTCTC -3′

TaqMan® PCR was performed on a StepOne Real Time PCR System (Applied Biosystems). To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2 x 30 µl) for each sample.

2.5.4 Sample quantification

Data from Taqman® PCR runs were collected with StepOne™ Software v 2.0.

Ct (cycle threshold) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values were used to create standard curves to serve as a basis for calculating the absolute amounts of mRNA in total RNA.

2.6 Calculation and statistical analysis

The data were statistically compared using one-way analysis of variance (ANOVA). The level of statistical significance was set at $P< 0.05$.

2.7 Sea bass PepT1 in silico analysis

The amino acid sequence of sea bass PepT1 was analyzed using the open reading frame (ORF) finder program which is available at NCBI (http://www.ncbi.nlm.nih.gov). Nucleotide sequences were compared with other sequences available at the GenBank database using the BLAST algorithm (Altschul et al., 1997). Sequences were aligned using ClustalW program (www.ebi.ac.uk/clustalw), and Multiple Sequence Alignments Editor & Shading Utility, GeneDoc, version 2.6.002 (www.psc.edu/biomed/genedoc). Putative transmembrane domains were predicted using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), which is part of the Simple Modular Architecture Research Tool (SMART; at http://www.expasy.org/prosite/). Potential N-glycosylation, cAMP/cGMPdependent protein kinase and protein kinase C recognition sequences were identified using the PROSITE 20.40 computational tools (http://www.expasy.org/prosite/).

A phylogenetic tree was constructed on PepT1 amino acid sequences of sea bass and other teleost and higher vertebrate species. The phylogenetic reconstruction was generated by the neighbour-joining method (Saitou and Nei, 1987), as implemented in MEGA 4.0 (Tamura et al., 2007).

GenBank accession numbers for amino acid sequence comparisons are: AAY17354 [Atlantic cod PepT1 (Rønnestad et al., 2007)]; ABV82968 (China rockfish Pept1); AAQ65244 [zebrafish PepT1 (Verri et al., 2003)]; AAK39954 (chicken PepT1); AAO16604 (turkey PepT1); AAA17721 [rabbit PepT1 (Fei et al., 1994)]; NP_001003036 (dog PepT1); AAO43094 (pig PepT1); NP_001092848 (cattle PepT1); AAA63797 (human PepT1); NP_001028071 (macaque PepT1); BAA09318 [rat PepT1 (Miyamoto et al., 1996)]; NP_444309 (mouse PepT1); AAK14788 (sheep PepT1).

3. Results

3.1 DNA sequencing and bioinformatic analysis

Obtained sequences have been analysed by bioinformatic programs available in public database. A BlastN search was performed on the complete, nonredundant GenBank nucleotide database for the orthologues of the considered genes, Δ6 desaturase, lipin and PepT1, in other fish species, using the web server at NCBI (http:// www.ncbi.nlm.nih.gov/BLAST/). A multiple sequence nucleotide alignment was performed on coding sequences for each target gene, with ClustalW algorithm, located at the European Bioinformatic Institute (http://www.ebi.ac.uk/clustalw). Primers were designed with Primer3 utility (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Primers design was based on identified regions of strong nucleotide conservation. All the alignments presented several conserved regions within the sequence where primers could be reasonably designed. Using the designed primer sets, the full-length cDNAs for Δ6 desaturase and PepT1 were isolated, while a cDNA fragment of lipin was obtained. A complete coding sequence of sea bass PPARγ (accession no. AY590303) was already present in database, at the beginning of this study. All the coding sequences identified were then deposited in GenBank with a specific accession number.

3.1.1 Δ6 desaturase cDNA sequence

Primer design was based on the alignment of four teleost Δ6 desaturase coding sequences available on the NCBI GenBank database: Atlantic cod (accession no. AAY46796), gilthead seabream (accession no. AAL17639), Atlantic salmon (accession no. NP_001117047) and rainbow trout (accession no. NP_001117759).
Two cDNA fragments were obtained in two step of PCR using as template cDNA intestine and these primers: the first one using Δ6-sense1 + antisense1 and the second using Δ6-sense2 + antisense2 (Table 2.1, Fig. 2.2). Then, the transcript was completed by 5'- and 3'-RACE. The full-length Δ6 desaturase cDNA consists of 2162 bp, comprising a 5'-untranslated region (351 bp), an Open Reading Frame (ORF) (1338 bp), and a 3'-untranslated region (472 bp), including the possible polyadenylation signal (AATAAA) (Fig. 3.1).

The deduced amino acid sequence shows that sea bass Δ6 desaturase is 445 amino acid long with a predicted molecular mass of approximately 51.7 KDa. The entire sequence was finally deposited in GenBank under the accession no. EU647692.

The amino acid sequence identity among the species was calculated using the open reading frame. Sea bass Δ6 desaturase protein shows a high percentage of identity both with other species of teleost (70-80%) and with orthologous proteins of higher vertebrates (60%). The % similarity for alignments of Δ6 desaturase for different species, including the sizes of protein, are presented in Table 3.1.

```
cgtgcttaatatcgatacagacaaatgatgccaactctatatgtgaaaatccgccctatt     60
tcagcaaacagcctcctggttgtcggatttcatcatcaattattagtgaagatgttgctc    120
tgctctttgtcttacttcacttgtgttacaatcagttgggtgtttacagtaaagagagca    180
acaggttaaaaccctcctcctctgaatgctgctccctctgcacaatctgcggctgcagac    240
tgtcagaggggtgttaacagacctttccacatccaggtgtcaaacttgtggctggctgg    300
atctttttagtgtaggcttcaggtggatccaggccagagacagcagtgaggatggaggt    360
                                                  M  G  G
ggaggccaactgacggagccaggagagtcgggcagcaggcgagctggaggtgtttacacc    420
 G  G  Q  L  T  E  P  G  E  S  G  S  R  R  A  G  G  V  Y  T
tgggaggaggtgcagagccactgcaacaggaatgaccagtggctggtcattgatcgaaag    480
 W  E  E  V  Q  S  H  C  N  R  N  D  Q  W  L  V  I  D  R  K
gtttatgacatcacccattgggccaaagggcacccaggagggcaccgtgtcatcagccac    540
 V  Y  D  I  T  H  W  A  K  G  H  P  G  G  H  R  V  I  S  H
tatgctggagaggatgccacggaggccttcactgcttttcatcccaatttaaagtttgtg    600
 Y  A  G  E  D  A  T  E  A  F  T  A  F  H  P  N  L  K  F  V
caaaagtttctgaagcccctgctgattggagagctggcagcaacagagcccagccaggac    660
 Q  K  F  L  K  P  L  L  I  G  E  L  A  A  T  E  P  S  Q  D
cgaaacaaaaatgcagcaattatacaggatttctacactttacgtgcgcaggcagagagc    720
 R  N  K  N  A  A  I  I  Q  D  F  Y  T  L  R  A  Q  A  E  S
gagggtctgtttaaagctcagcctttgttcttctgcctccacctgggtcacatcctgctg    780
 E  G  L  F  K  A  Q  P  L  F  F  C  L  H  L  G  H  I  L  L
ttggaggccctcgcctggctcatcatctggctctggggaaccagctggactctgacattt    840
 L  E  A  L  A  W  L  I  I  W  L  W  G  T  S  W  T  L  T  F
ttgtgctcgatcatgctggcaactgctcagtcgcaggccggatggctgcagcacgacttt    900
 L  C  S  I  M  L  A  T  A  Q  S  Q  A  G  W  L  Q  H  D  F
ggccacctgtctgtcttcaagaagtccagctggaatcacatgttgcacaagtttgtcatc    960
 G  H  L  S  V  F  K  K  S  S  W  N  H  M  L  H  K  F  V  I
ggtcatctaaagggagcctctgccaactggtggaatcatcggcatttccagcatcacgct   1020
 G  H  L  K  G  A  S  A  N  W  W  N  H  R  H  F  Q  H  H  A
aaacccaacatcttcagtaaggacccagatgtcaacatgttgcacatcttcgtagttgga   1080
 K  P  N  I  F  S  K  D  P  D  V  N  M  L  H  I  F  V  V  G
gccactcaaccagtagagtatggcataaagaagatcaaatatatgccctatcatcaccaa   1140
 A  T  Q  P  V  E  Y  G  I  K  K  I  K  Y  M  P  Y  H  H  Q
caccagtacttccttcttgttggaccaccgctgctcattccagtttacttccacattcag   1200
 H  Q  Y  F  L  L  V  G  P  P  L  L  I  P  V  Y  F  H  I  Q
attatacgcaccatgatttcccgccgtgactgggtggatctggcttggtctatgtcttac   1260
 I  I  R  T  M  I  S  R  R  D  W  V  D  L  A  W  S  M  S  Y
taccttcgctacctgtgctgttatgtacccttgtatggcctgtttggctcgttggcgctc   1320
 Y  L  R  Y  L  C  C  Y  V  P  L  Y  G  L  F  G  S  L  A  L
atcagtttcgtcaggtttttggagagtcactggtttgtgtgggtgactcagatgaatcat   1380
 I  S  F  V  R  F  L  E  S  H  W  F  V  W  V  T  Q  M  N  H
ctgccgatggacatcgaccacgagaagcaccatgactggctgaccatgcagttacaagcc   1440
 L  P  M  D  I  D  H  E  K  H  H  D  W  L  T  M  Q  L  Q  A
acctgtaatattgagaagtcctctttcaacgactggttcagcggacacctcaactttcaa   1500
 T  C  N  I  E  K  S  S  F  N  D  W  F  S  G  H  L  N  F  Q
atcgaacaccatttgtttcctacaatgccgcgccacaactaccacctggtggcgccgctg   1560
 I  E  H  H  L  F  P  T  M  P  R  H  N  Y  H  L  V  A  P  L
gtccatgcactgtgtgagaaacatgggattccttaccaggtgaagacgatgtggcaaggc   1620
 V  H  A  L  C  E  K  H  G  I  P  Y  Q  V  K  T  M  W  Q  G
cttgttgatgttatcaggtcactgaaaaactcaggggacctctggcttgatgcatatctc   1680
 L  V  D  V  I  R  S  L  K  N  S  G  D  L  W  L  D  A  Y  L
cataaatgaccaattgtattctctaccctgtacctaaaaggagtgatgtttttcttctct   1740
 H  K  -
tctgcatcatacattgattgtatcagtttggttttataatccagttgatagtgtgggaat   1800
gatcttttcttatcgttggtgttatagtttatagtcttttcagattctgtgcagtatttt   1860
tagtgctcacaggatttttctctaaattgccttacagtatcttgatcatcagtggtatgg   1920
tgttatcaatacaattgtgaaaattgatttgtgagttatttaaggtgatgtttttttctt   1980
tatttacaatgtgatacagtttgaacaataaaacagacaaattacacatcctgttttcct   2040
gtttaactgaggatgaaaatgttctccacttaattaaattttcttctttctgtcaaactt   2100
ctttacttgcaactctacgtcttacaataaaggaactgtattgacaaaaaaaaaaaaaaa   2160
                                                        aa   2162
```

Figure 3.1 The nucleotide sequence of sea bass Δ6 desaturase, with the deduced amino acids shown below the sequence in single-letter code. Nucleotide are numbered to the left. The start (ATG) and end (TGA) codons are indicated in bold; the polyadenylation signal (AATAAA) is indicated in bold italic.

Table 3.1 Shared identities (%) between Δ6 desaturase coding sequences in different teleosts, amphibian, avian and mammalian species.

Gene	Specie	Acc. no.	Protein size (aa)	Identity with *D. labrax* (%)
Δ6 desaturase	*Dicentrachus labrax*	ACD10793	445	-
	Sparus aurata	AAL17639	445	94
	Rachycentron canadum	ACJ65149	442	87
	Scophthalmus maximus	AAS49163	445	83
	Gadus morhua	AAY46796	447	81
	Oncorhynchus masou	BAB63440	454	76
	Oncorhynchus mykiss	NP_001117759	454	75
	Oreochromis niloticus	BAB62850	445	76
	Salmo salar	NP_001117047	454	76
	Danio rerio	AAG25710	444	68
	Xenopus laevis	AAI12949	446	66
	Gallus gallus	ABR24806	440	68
	Coturnix japonica	ABC87787	407	67
	Homo sapiens	AAD20018	444	65
	Mus musculus	AAD20017	444	65
	Rattus norvegicus	BAA75496	444	64
	Bos taurus	AAI23736	444	65

3.1.2 Lipin cDNA sequence

Lipin primers were designed on the alignment of orthologues in western clawed frog, *Xenopus tropicalis* (accession no. NP_001072495); rat, *Rattus norvegicus* (accession no. NP_001012111); mouse, *Mus musculus* (accession no NP_766538), and human, *Homo sapiens* (accession no. NP_663731). The sea bass lipin partial coding sequence was obtained by RT-PCR from adipose tissue reverse transcribed with the pair primers Lipin sense1 + antisense1 (Table 2.1, Fig. 2.3). The sequence we isolated and deposited in the database with accession no. EU644089 corresponds to a portion of ORF of 379 bp, and virtually gives a translated protein of 126 amino acids, as shown in figure 3.2. Sea bass lipin amino acid sequence presents a high percentage of identity (90%) with sequences of other vertebrate available in the database. The values of identity with the orthologous sequences, expressed in percentage, are presented in Table 3.2.

```
taccacaaagtgagccagaatggatataaattcatgtactgctcggcgagggccattggc   60
 Y  H  K  V  S  Q  N  G  Y  K  F  M  Y  C  S  A  R  A  I  G
Atggctgatatgactcgaggctacttgcactgggtcaatgagaggggaaccatgctgcca  120
 M  A  D  M  T  R  G  Y  L  H  W  V  N  E  R  G  T  M  L  P
Gtgggcccagtgctgctgagccccagcagccttttttctgctttgcacagggaggtgatt  180
 V  G  P  V  L  L  S  P  S  S  L  F  S  A  L  H  R  E  V  I
Gagaagaaaccagagaagtttaagatcgagtgtctctcagacatcaagcatcttttctac  240
 E  K  K  P  E  K  F  K  I  E  C  L  S  D  I  K  H  L  F  Y
Ccaaacacagaacctttctacgctgcttttggcaacagagctacggatgtgtattcctac  300
 P  N  T  E  P  F  Y  A  A  F  G  N  R  A  T  D  V  Y  S  Y
Aaggaggtgggtgttcctctgaacaggattttcactgtcaatcccaaaggggagctgata  360
 K  E  V  G  V  P  L  N  R  I  F  T  V  N  P  K  G  E  L  I
                                              caggagcacgcaaagacca  379
                                               Q  E  H  A  K  T
```

Figure 3.2 The nucleotide sequence of sea bass lipin, with the deduced amino acids shown below the sequence in single-letter code. Nucleotide are numbered to the left.

Table 3.2 Shared identities (%) between lipin coding sequences in different vertebrate species.

Gene	Specie	Acc. no.	Protein size (aa)	Identity with *D. labrax* (%)
Lipin	*Dicentrachus labrax*	ACD03132	126	-
	Danio rerio	AAI63254	894	95
	Xenopus tropicalis	NP_001072495	842	93
	Gallus gallus	XP_419957	1252	91
	Homo sapiens	NP_663731	890	88
	Mus musculus	NP_766538	891	88
	Ratus norvegicus	NP_001012111	924	88

3.2 Sea bass PepT1 cDNA sequence

Primer design was based on the alignment of three teleost PepT1 coding sequences available on database: Atlantic cod (accession no. AY621934), China rockfish (accession no. EU160494), and zebrafish (accession no. AY300011).

Two cDNA fragments were obtained using these primers: the first one using PepT1 sense1 + antisense1 ant the second using PepT1 sense2 + antisense2 (Table 2.1, Fig. 2.4). Then, by connecting the sequences of the partially overlapping clones, a partial coding sequence (~1000 bp) for sea bass pepT1 was determined. The full-length cDNA for PepT1 was subsequently isolated by 5'- and 3'-RACE and deposited in GenBank under the accession no. FJ237043. The respective nucleotide and deduced amino acid sequences are shown in figure 3.3 and 3.4.

The sea bass PepT1 cDNA consists of 3014 bp, comprising a 5'-untranslated region (101 bp), an open reading frame (2184 bp), and a 3'-untranslated region (729 bp), including the possible polyadenylation signal (AATAAA) (Fig. 3.3 and 3.4).

The deduced amino acid sequence shows that sea bass Pept1 is 727 amino acid long with a calculated molecular mass of approximately 81 kDa. The transmembrane domains of sea bass PepT1 protein, predicted with THMM program (http://www.cbs.dtu.dk/services/) are presented in figure 3.4 and 3.5. Sea bass PepT1 adopt a 12 transmembrane domain structure, with intracellular amino- and carboxyl-terminus, a large extracellular loop between the membrane spanning helices (MSH) 9 and 10, and an elongated intracellular loop between MSH 6 and 7.

In Figure 3.4, the deduced amino acid sequence of sea bass PepT1 (accession no. ACI49693) is aligned with the PepT1 related protein of Atlantic cod (accession no. AAY17354); zebrafish (accession no. AAQ65244); chicken, *Gallus gallus* (accession no. NP_989696); rabbit, *Oryctolagus cuniculus* (accession no. AAA21335); rat (accession no. NP_476462), and human (accession no. NP_005064). Amino acids are designated by single-letter codes and are numbered to the right side; dots indicate conserved residues in sea bass PepT1, and dashes indicate gaps introduced to facilitate alignment. The numbered straight lines on top of the sequence alignment give the positions of the MSH in sea bass. Three putative extracellular N-glycosylation sites (inside a box), and (Asn^{120}, Asn^{498}, Asn^{513}), and four putative intracellular cAMP/cGMP- dependent protein kinase A (Ser^{363}, Ser^{694}, Ser^{702}, Ser^{713}) (black triangle) are indicated along the sequences where found (Fig. 3.4). The

proposed 'PTR2 family proton/oligopeptide symporters signature 1' motif (PROSITE pattern: PS01022; amino acid residues 73–97 in sea bass PepT1) and 'PTR2 family proton/oligopeptide symporters signature 2' motif (PROSITE pattern: PS01023; amino acid residues 166-178 in sea bass PepT1) are highlighted in black. Individual amino acid residues identified by site-directed mutagenesis in PepT1 proteins from various mammalian species and found to be relevant in determining the functional characteristics of the protein are indicated in light grey (Fig. 3.4).

```
                          cttcgtgtcttggatccggacagggttcactagtctctttg    41
tgtctttttgctgaaggattcagtcggagaagggacctgagatcgacacacacagcagcc    101
atggcagacgggaagaagtcaaaaagcgctactgcctgtggctacccaataagcatcttt    161
 M  A  D  G  K  K  S  K  S  A  T  A  C  G  Y  P  I  S  I  F
ttcattgtggtcaatgagttctgcgagcgtttctcctactatggcatgcgagccgtgctg    221
 F  I  V  V  N  E  F  C  E  R  F  S  Y  Y  G  M  R  A  V  L
gtgctgtactttaagtacttcctgaggtgggatgatgacttcgccaccactatctaccac    281
 V  L  Y  F  K  Y  F  L  R  W  D  D  D  F  A  T  T  I  Y  H
acctttgtggctctctgctacctgacacccatcctgggagccattgtggcagattcatgg    341
 T  F  V  A  L  C  Y  L  T  P  I  L  G  A  I  V  A  D  S  W
ctcggcaagttcaagaccattgtttacctgtccatcgtttatacgctggggcagattgtc    401
 L  G  K  F  K  T  I  V  Y  L  S  I  V  Y  T  L  G  Q  I  V
atggcaataagtgctatccatgacatcacagattcaaacaaagacggcattcccgacaac    461
 M  A  I  S  A  I  H  D  I  T  D  S  N  K  D  G  I  P  D  N
atgaccttccacgtagctctgtctatggtgggtttgatccttattgccctgggaacggga    521
 M  T  F  H  V  A  L  S  M  V  G  L  I  L  I  A  L  G  T  G
ggcatcaaaccctgcgtggctgcctttggtggagaccagtttgaggaccatcaggagaag    581
 G  I  K  P  C  V  A  A  F  G  G  D  Q  F  E  D  H  Q  E  K
cagagaagtaccttcttctccatcttctacctgtcaatcaatgctggcagcctgctgtcc    641
 Q  R  S  T  F  F  S  I  F  Y  L  S  I  N  A  G  S  L  L  S
actgtcatcacccccatcctcagagctcaggaatgtggcattcacacccagcagaagtgc    701
 T  V  I  T  P  I  L  R  A  Q  E  C  G  I  H  T  Q  Q  K  C
taccctctggcctttggtgtccccgctgctctcatggtggtagctctgattgtgttcatt    761
 Y  P  L  A  F  G  V  P  A  A  L  M  V  V  A  L  I  V  F  I
gttggaagcgggatgtacaataagactgcccctcaaggcaacatcattgtgcaagtctgc    821
 V  G  S  G  M  Y  N  K  T  A  P  Q  G  N  I  I  V  Q  V  C
aaatgcatcgggtttgctattaagaaccgcttcaggcatcgtgctcgtgaacacccttgc    881
 K  C  I  G  F  A  I  K  N  R  F  R  H  R  A  R  E  H  P  C
agaacccactggatggactgggctgaggagaaatacgataaactcctgattgcgcaggtg    941
 R  T  H  W  M  D  W  A  E  E  K  Y  D  K  L  L  I  A  Q  V
aagatggtgctgaaggtgctcttcctctacatccctcttcccatgttctgggctcttttt   1001
 K  M  V  L  K  V  L  F  L  Y  I  P  L  P  M  F  W  A  L  F
gaccagcagggctcaagatggaccctccaggcgaccaccatggacggcgactttggagct   1061
 D  Q  Q  G  S  R  W  T  L  Q  A  T  T  M  D  G  D  F  G  A
ctcataatccagcccgatcagatgcagactgtcaaccctatcctgatcctggttttggtg   1121
 L  I  I  Q  P  D  Q  M  Q  T  V  N  P  I  L  I  L  V  L  V
ccaatcatggacagtttggtctacccgctgatttccaagtgcaaattaaacttctctccg   1181
 P  I  M  D  S  L  V  Y  P  L  I  S  K  C  K  L  N  F  S  P
ctgaagaggatgactgtggggatgttccttgctgctctagccttcatcgctgccgccctg   1241
 L  K  R  M  T  V  G  M  F  L  A  A  L  A  F  I  A  A  A  L
gtccagatacagattgatcaaaccctgcctaagttcccatcaagcactgtaggccaagcg   1301
 V  Q  I  Q  I  D  Q  T  L  P  K  F  P  S  S  T  V  G  Q  A
```

```
aagttcatcaacatggttaacagagcgttgaacattaatgctggacccaacagctttacc   1361
 K  F  I  N  M  V  N  R  A  L  N  I  N  A  G  P  N  S  F  T
ttggagtcttataaggccaatgaggaatactttaactttaatggaccatttaagctgaat   1421
 L  E  S  Y  K  A  N  E  E  Y  F  N  F  N  G  P  F  K  L  N
ctggggtctggaaatgctttttaggcaacataccagctggcactcgggcgaccattgtc   1481
 L  G  S  G  N  A  F  L  G  N  I  P  A  G  T  R  A  T  I  V
atcattcaggatgggaccaaacccagacctacacagttcagagacatcaaatcaaagccg   1541
 I  I  Q  D  G  T  K  P  R  P  T  Q  F  R  D  I  K  S  K  P
gaacagggcactaacgctatcagatttcttaatggttttggctcggttttgaatgcaaca   1601
 E  Q  G  T  N  A  I  R  F  L  N  G  F  G  S  V  L  N  A  T
gtgggtaccctggagtttgggaacaccatcaccaacaacatgtcagaatatatttcggta   1661
 V  G  T  L  E  F  G  N  T  I  T  N  N  M  S  E  Y  I  S  V
ccacagggaaatgcacagttccatatcagggacaatagcggacaagagtgtgtctacact   1721
 P  Q  G  N  A  Q  F  H  I  R  D  N  S  G  Q  E  C  V  Y  T
cagaaactgggcttcggcagctcgtacactttgatcatcccgccaactttcgcatttgga   1781
 Q  K  L  G  F  G  S  S  Y  T  L  I  I  P  P  T  F  A  F  G
ccaaattgtgaaaacagcatccagccagtggttgacatcaagcctaacaccatccacatg   1841
 P  N  C  E  N  S  I  Q  P  V  V  D  I  K  P  N  T  I  H  M
gcctggcagattcctcagtatttcctcatcactgcaggagaggtggtcttctctgtcacc   1901
 A  W  Q  I  P  Q  Y  F  L  I  T  A  G  E  V  V  F  S  V  T
ggactggagttctcctactcacaggcacccagcaacatgaagtctgtgctgcaggctggt   1961
 G  L  E  F  S  Y  S  Q  A  P  S  N  M  K  S  V  L  Q  A  G
tggctgctaactgttgctgtcggtaacatcattgtgctcattgtcgctgaggctgcaacg   2021
 W  L  L  T  V  A  V  G  N  I  I  V  L  I  V  A  E  A  A  T
ctcccagatcagtgggccgagtacatcctcttcgcctctctgctgatcttagtgtgcatc   2081
 L  P  D  Q  W  A  E  Y  I  L  F  A  S  L  L  I  L  V  C  I
atctttgccgtcatggcctatttctacacctaccttgacccagccaagatagaagccgag   2141
 I  F  A  V  M  A  Y  F  Y  T  Y  L  D  P  A  K  I  E  A  E
tttgcccacgaggagcctgaagataaggagaagaggaagagtttggagatggccaagaag   2201
 F  A  H  E  E  P  E  D  K  E  K  R  K  S  L  E  M  A  K  K
gactcggttgagcaccacaaggaggacagaaggagttctgactccagctctgacgaggag   2261
 D  S  V  E  H  H  K  E  D  R  R  S  S  D  S  S  S  D  E  E
gaaaccaaacagaccaagatc**taa**agtgatccctccctcgtagacgtggttctgtgtttt   2321
 E  T  K  Q  T  K  I  -
atatctgagagtgcctgtgccatgaagtttttccttttttgggggggtcattttgtcaat   2381
gcagtctgttgcactgctggacatggagtggttagaatgaatggatgacctgagtgacat   2441
ggtgctgaagttggacgaacacagacgcagagcttagatggttaagctcttggtagacag   2501
tgttagtggagaatcttggcagggataattcaaacactcttgtcaccatttcggctcaaa   2561
atattgagagaatattccttttttagaggttatacgtataattaaaatccctggatctta   2621
agtggttagtaatttctgctttggctgttgtaaattagttttacaatgatttattttgc   2681
actgtgtagcaacatctttatgtaattttagtttcataggcttttgtactgtactggagt   2741
acgcatacaatgaaaccattccaacatttgtatttatttccaagcacagtgttgtatgta   2801
ttgtaatatattgccactgttttatatgtatatgatgtcctcatctgctccagaggaaaa   2861
atctaacagcaaaagactgtttattcagtcagaaaatgtttaatcatattccatatccaa   2921
ccctcagagttcattctggcctgggacaatggctgctcaccaaggtcttc***aataaa***gatt   2981
                          tgtcaacaaacgtaaaaaaaaaaaaaaaaaaaa   3014
```

Figure 3.3 The nucleotide sequence of sea bass PePT1 with the deduced amino acids shown below the sequence in single-letter code. Nucleotide are numbered to the left. The start (ATG) and end (TAA) codons are indicated in bold; the polyadenylation signal (AATAAA) is indicated in bold italic.

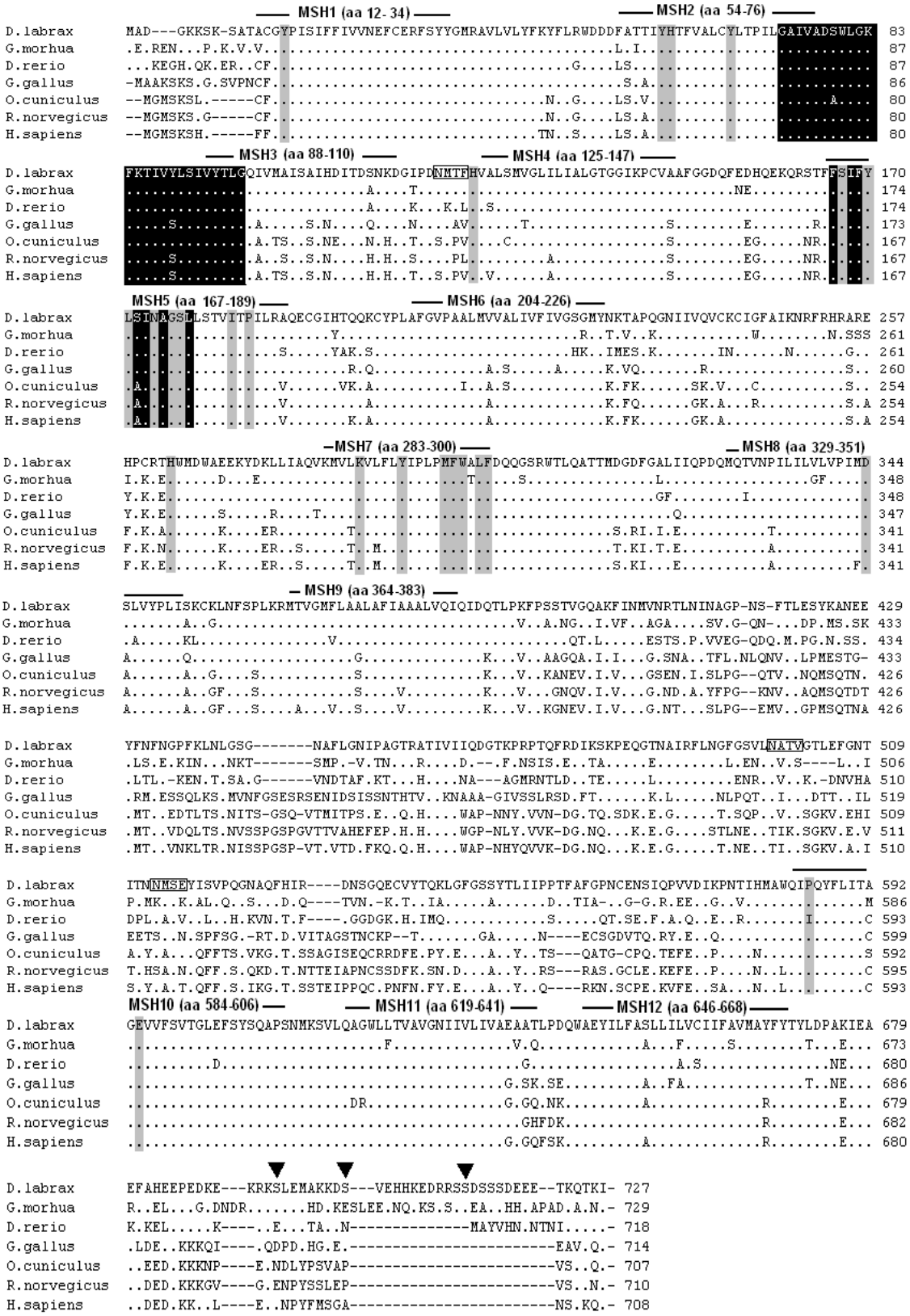

Figure 3.4 Alignment of the deduced amino acid sequence of sea bass PepT1 with the PepT1 related protein of other vertebrates (see *section 3.2* for explanations).

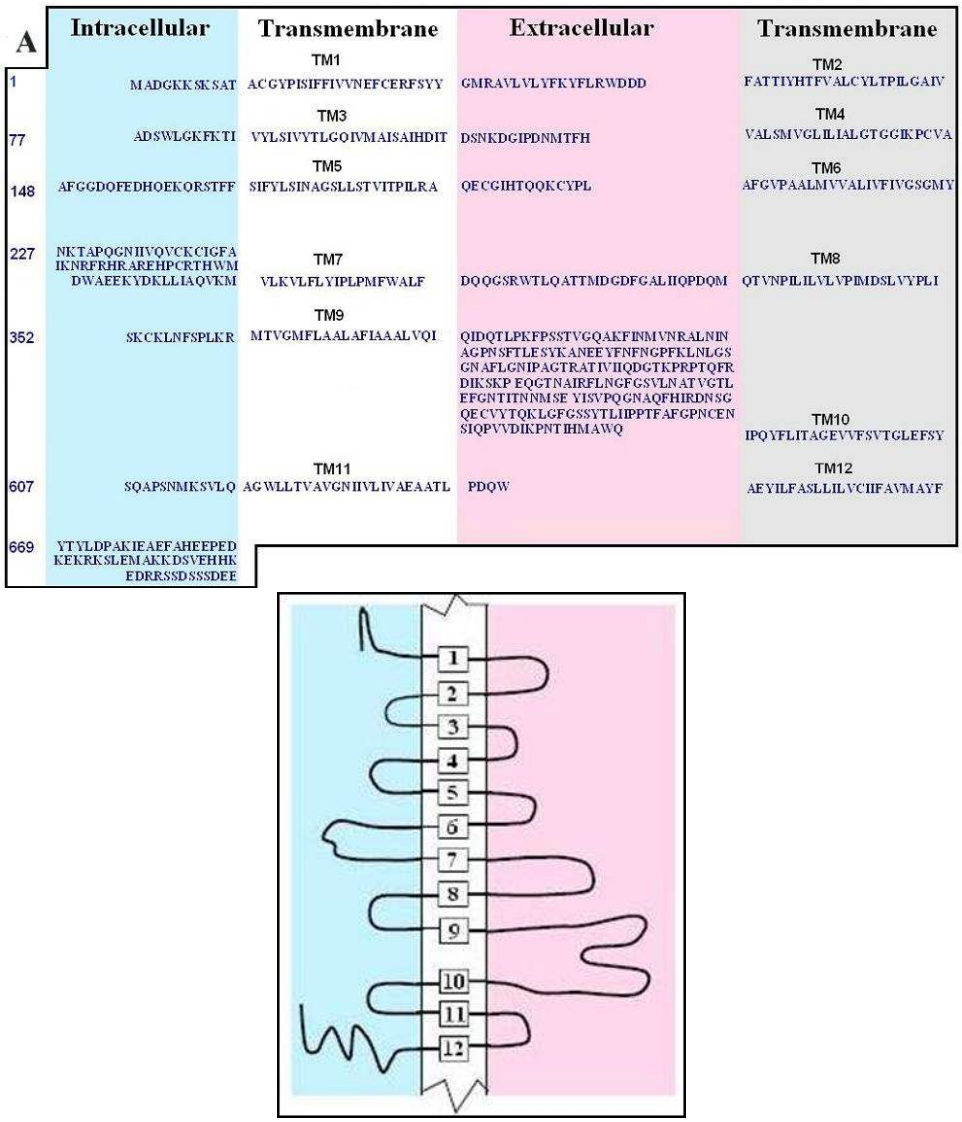

Figure 3.5 Membrane spanning helices in sea bass PepT1 protein. Putative membrane topology is indicated by the blue (intracellular), white (transmembrane, N terminal near the intracellular face), pink (extracellular), and gray (transmembrane, N terminal near the extracellular face) regions. Residues are shown in dark blue letters and are numbered to the left.

3.2.1 Sea bass PepT1 phylogenetic analysis

The % similarities for alignments of PepT1 for different species, including the sizes of protein, are presented in Table 3.3. The amino acid sequence identity among the species was calculated using the open reading frame. Sea bass PepT1 showed the highest sequence homology with teleosts (Atlantic cod 68 %; zebrafish 65 % China rockfish 64 %) and avian species (chicken 61%; turkey: 63%), and lower

homology with mammalian species (dog 59%; rabbit and rat 57%; mouse, pig, cattle, sheep, rhesus monkey and human 58 %).

Alignment of the amino acid sequence of sea bass PepT1 with that of other teleost, avian, and mammalian species is shown in Fig. 3.4. In the same figure are also indicated the positions of the MSH predicted with THMM program and the highly conserved amino acids.

Table 3.3 Shared identities (%) between PepT1 coding sequences in different teleosts, avian and mammalian species.

Gene	Specie	Acc. no.	Protein size (aa)	Identity with *D. labrax* (%)
PepT1	*Dicentrachus labrax*	ACI49693	727	-
	Gadus morhua	AAY17354	729	68
	Sebastes nebulosus	ABV82968	742	64
	Danio rerio	AAQ65244	718	65
	Gallus gallus	AAK39954	714	63
	Meleagris gallopavo	AAO16604	714	63
	Homo sapiens	AAA63797	708	58
	Canis lupus familiaris	NP_001003036	708	59
	Mus musculus	NP_444309	709	58
	Rattus norvegicus	BAA09318	710	57
	Sus scrofa	AAO43094	708	58
	Bos taurus	NP_001092848	707	58
	Ovis aries	AAK14788	707	58
	Oryctolagus cuniculus	AAA17721	707	57
	Macaca mulatta	NP_001028071	708	58

To analyze the evolutionary relationship of sea bass PepT1 with respect to other publicly available related genes in teleosts, avian, and mammalian species, we reconstructed a phylogenetic tree (Fig. 3.6). The clustering pattern provides evidence that sea bass PepT1 is grouped with high bootstrap support in the lineage of other teleosts, sharing the highest homology with Atlantic cod PepT1, whereas the avians (chicken, turkey), and the mammalians (human, rhesus monkey, dog, rat, mouse, cattle, pig, sheep) are grouped into two other distinct lineages.

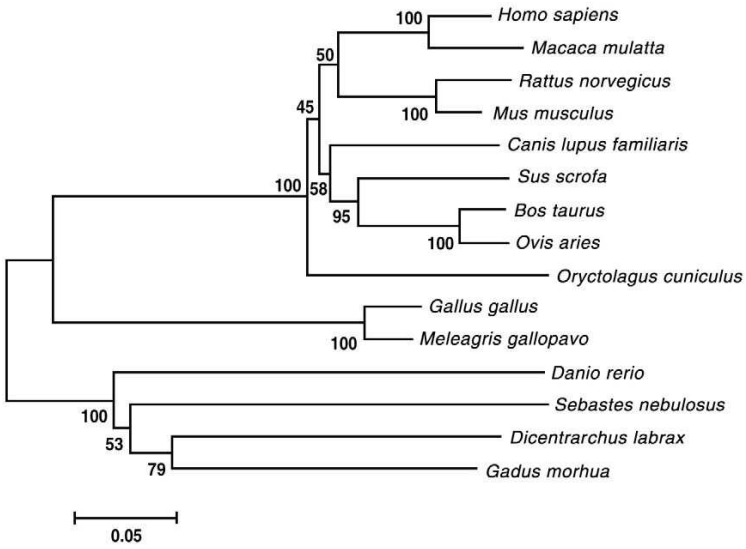

Figure 3.6 Unrooted phylogenetic tree depicting the evolutionary relationship of vertebrate PepT1 transporters. The unrooted tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) based on the alignment of the complete amino acid sequences of known vertebrate PepT1 transporters. Bootstrap values (1000 replicates) indicating the occurrence of nodes is reported above each branch in the figure.

3.3 Fish growth and feeding rates

The growth and condition factor data are reported in figure 3.7 and 3.8, respectively. At the onset of fasting period the mean body weight of the sea bass was 119.34 ± 15.27 g for the control group and 118.41 ± 18.37 g for the fasted group, whereas the mean condition factors were 1.56 ± 0.16 and 1.57 ± 0.21, respectively. After 35 days of fasting the mean body weight and condition factor of fasted fish were significantly lower than the fed control($P<0.05$). During the subsequent refeeding period, previously unfed fish were able to increase the body weight sufficiently to overcome the weight loss imposed by the 5-weeks feed restriction.

Fish that had experienced feed deprivation exhibited higher feeding rates than ad libitum fed controls during the first two weeks of refeeding (Fig. 3.9) ($P< 0.05$). Refeeding of sea bass after 35 days of starvation was marked by hyperphagia as early as the first day. The hyperphagic period was however shorter than the fasting period.

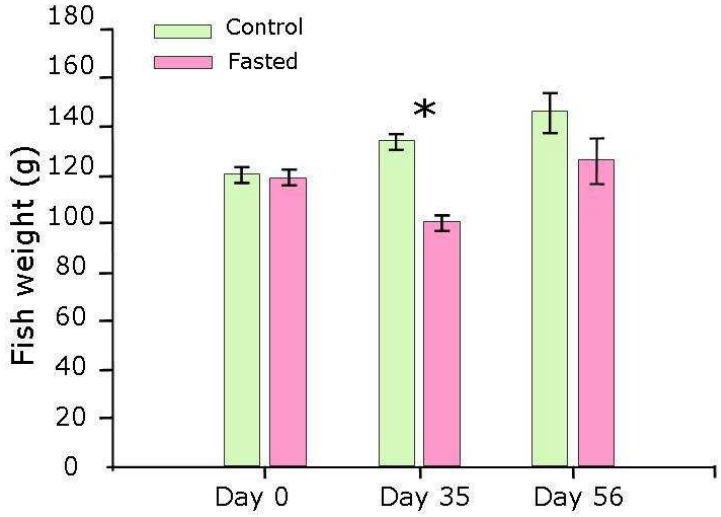

Figure 3.7 Mean body weight of sea bass control and fasted group. Fish were weighted before fasting (Day 0), at the end of fasting (35 days), and then at 21 days following refeeding. Difference were determined by one-way analysis of variance (ANOVA).
(*) indicates significantly different means (P<0.05).

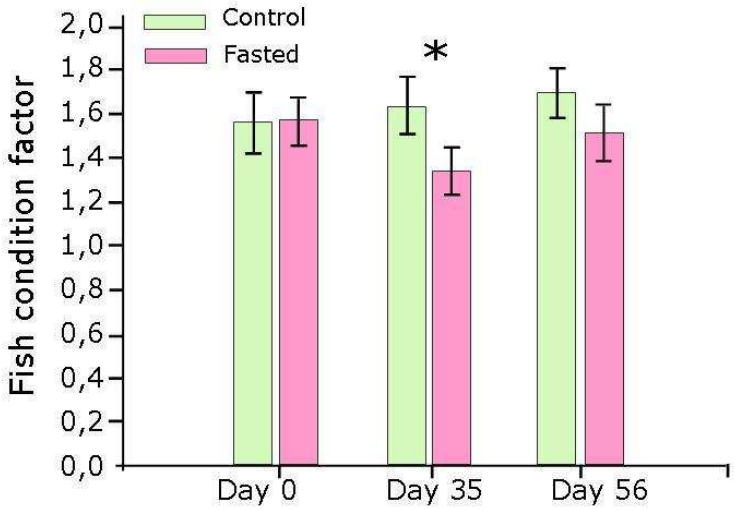

Figure 3.8 Condition factor (K= body weight x 100 / standard body length^3) of sea bass control and fasted group. Fish were measured before fasting (Day 0), at the end of fasting (35 days), and then at 21 days following refeeding. Difference were determined by one-way analysis of variance (ANOVA). (*) indicates significantly different means (P<0.05).

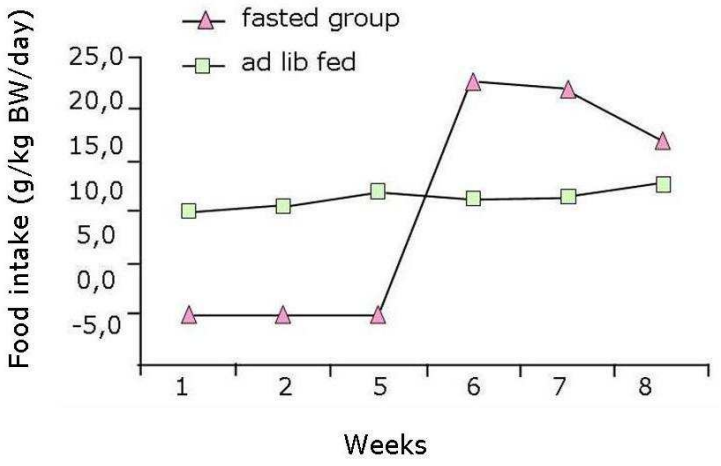

Figure 3.9 Weekly changes in mean daily food intake in group of sea bass subjected to fasting and refeeding (△) and in that fed ad libitum (□).

3.4 Creation of standard curves for absolute quantification of genes target

The correct template length including the T3 or T7 promoter was verified by 2% agarose gel electrophoresis. Quality and purity of cRNAs were confirmed by the ratio of absorptions at 280/260 nm, i.e., 1.8-2.0 (data not shown).

To obtain threshold cycle (Ct) values for the target genes, defined quantities at 10-fold dilutions of each gene cRNAs were subjected to a one-tube two-time real-time RT-PCR. The standard curves created for all gene considered were based on the linear relationship between the Ct value and the logarithm of the starting amount.

3.5 mRNAs copy number of genes involved in lipid metabolism in sea bass, during fasting and refeeding

The effects of fasting and subsequent refeeding were evaluated by real-time quantitative PCR on the expression levels of genes involved in lipid metabolism: Δ6 desaturase, lipin, PPARγ, measuring the transcript levels in liver and proximal intestine of sea bass.

3.5.1 Δ6 desaturase

The absolute mRNA levels of Δ6 desaturase in the liver and proximal intestine in response to the feeding trial are presented in figure 3.10A and B, respectively. In both tissues, the analysis showed significant changes in Δ6 desaturase expression levels during the experiment. In sea bass liver, fasting for 35 days significantly increased the amount of Δ6 desaturase transcripts ($P<0.05$), whereas 4 days without feed contributed to a significant decrease in transcript levels as compared to controls. The subsequent recovery from fasting was associated with a significant decrease in Δ6 desaturase mRNA transcript levels, compared to control fish, until the tenth day of refeeding. At the end of the refeeding period, the expression of Δ6 desaturase increased to levels comparable to control (Fig 3.10A).

Conversely, prolonged fasting caused a significant decrease in Δ6 desaturase transcripts in the proximal intestine as early as 4 days of fasting ($P<0.05$) (Fig. 3.10B). The recovery from fasting was associated with a significant increase in mRNA transcript levels, beyond control levels, until the end of the fourth day of refeeding, in support of compensatory growth. Subsequently, 10 day after refeeding the Δ6 desaturase mRNA copy number levels decrease compared to the previous time point tested, and then returned similar to controls at the end of 21 days of refeeding (Fig. 3.10B).

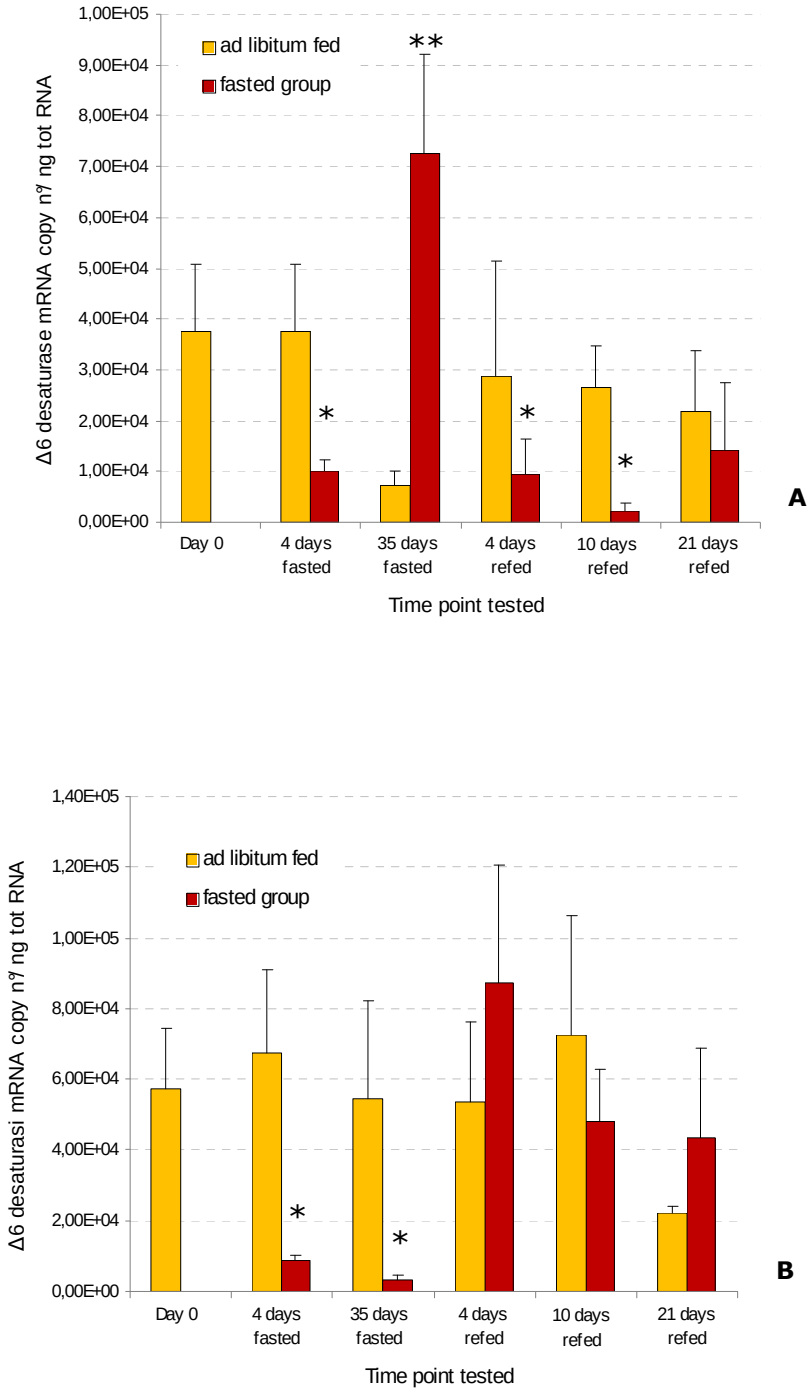

Figure 3.10 Expression levels of Δ6 desaturase measured by real-time PCR in sea bass liver (A) and proximal intestine (B), in the course of experiment. Δ6 desaturase mRNA copy number was normalized as ratio to 100 ng total RNA. Fish were sampled before fasting (Day 0), 4 days after fasting (4 days fasted), at the end of fasting (35 days fasted), and then sequentially at 4, 14, 21 days following refeeding. The means of five animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). (*) and (**) indicate significantly different means from controls, for each time point tested ($P<0.05$).

3.5.2 Lipin and PPARγ

The absolute mRNA levels of lipin and PPARγ followed a completely superimposable pattern. Fasting for 35 days significantly increased the amount of both gene transcripts ($P<0,05$), only in sea bass liver (Fig. 3.11A and 3.12A).

The recovery from fasting showed a significant decrease in lipin and PPARγ transcript levels as compared to control, at the end of 21 days of refeeding.

In the proximal intestine, the adopted food strategy did not affect lipin and PPARγ mRNA copy number levels (Fig. 3.11B and 3.12B). This result was in agreement with the membership of these two genes at the same transcriptional regulation system.

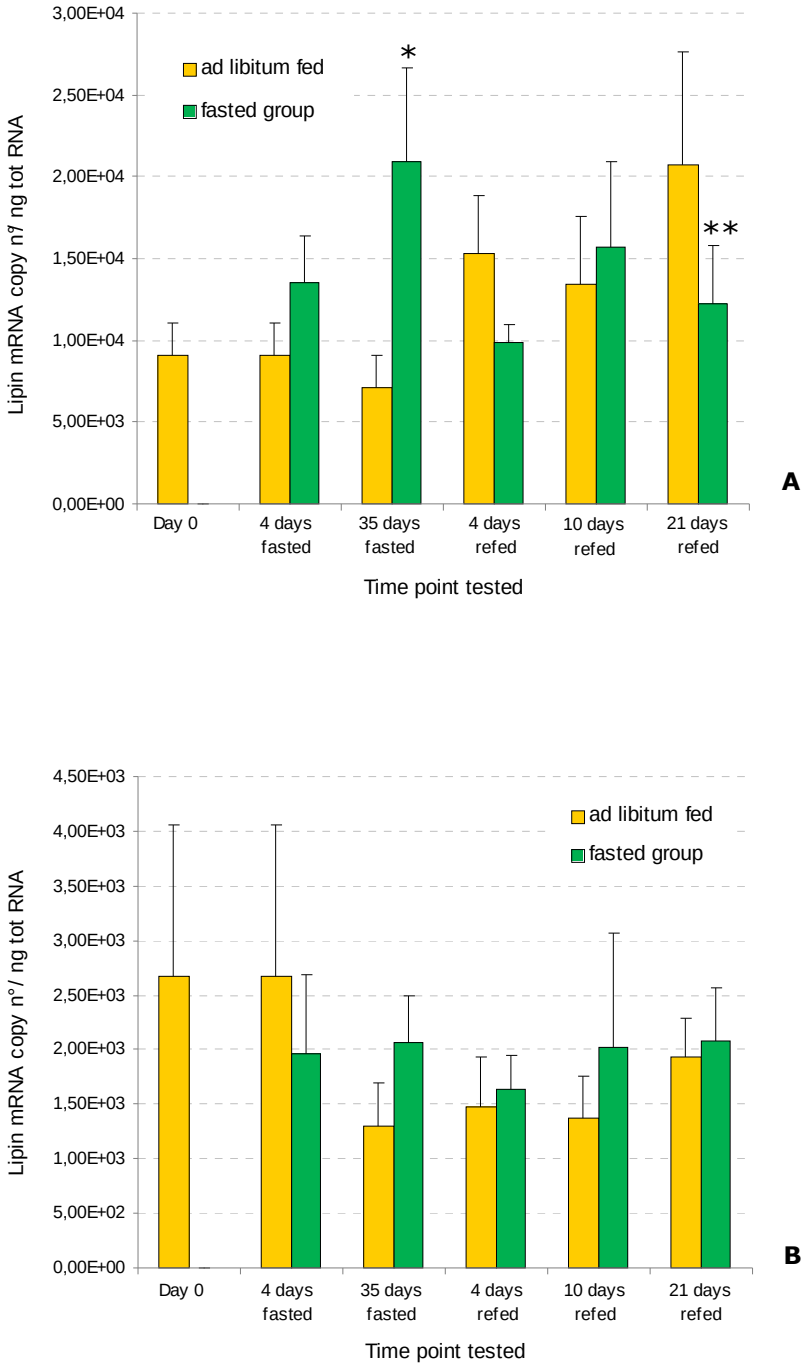

Figure 3.11 Expression levels of lipin measured by real-time PCR in sea bass liver (A) and proximal intestine (B), in the course of experiment. Lipin mRNA copy number was normalized as ratio to 100 ng total RNA. Fish were sampled before fasting (Day 0), 4 days after fasting (4 days fasted), at the end of fasting (35 days fasted), and then sequentially at 4, 14, 21 days following refeeding. The means of five animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). (*) and (**) indicate significantly different means from controls, for each time point tested ($P<0.05$).

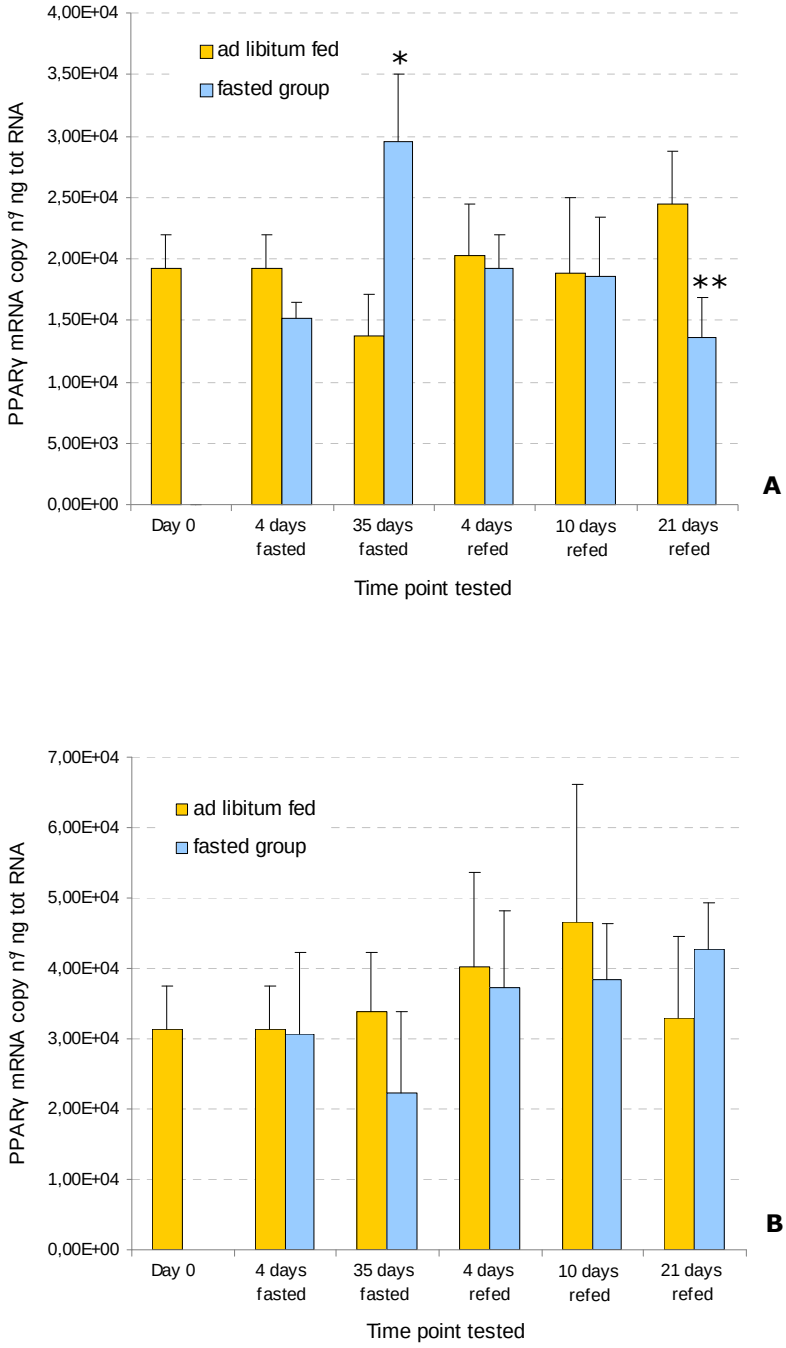

Figure 3.12 Expression levels of PPARγ measured by real-time PCR in sea bass liver (A) and proximal intestine (B), in the course of experiment. PPARγ mRNA copy number was normalized as ratio to 100 ng total RNA. Fish were sampled before fasting (Day 0), 4 days after fasting (4 days fasted), at the end of fasting (35 days fasted), and then sequentially at 4, 14, 21 days following refeeding. The means of five animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). (*) and (**) indicate significantly different means from controls, for each time point tested ($P<0.05$).

3.6 PepT1 mRNA copy number in sea bass tissues

Total RNA from sea bass tissues was subjected to real-time RT-PCR using the standard curve established for PepT1 cRNA to determine absolute amounts of PepT1 mRNA. This analysis revealed the following spatial distribution of PepT1 gene expression in sea bass digestive tract: high levels of expression in segments 1-3 (the first 3cm) of the proximal intestine, lower levels in pyloric caeca and intestinal segments 4-10, and very low levels in gastroesophageal junction, stomach fundus, pyloric antrum, most distal intestinal region (segments 9-10) and rectum (Fig. 3.13). The expression of sea bass PepT1 was also analysed in other tissues of adult fish. Here the mRNA copy number was in the range $1.08x10^2$ – $1.34x10^4$, so 100 fold less than that found along the intestine. We have presented the tissue expression levels of PepT1 including the segments of digestive tracts which are "invisible" in the Fig. 3.13, in another graph with a different scale (Fig. 3.14). As shown in this figure, the higher levels of mRNA copy number were found in the gills followed in a decreasing way by brain, heart, liver, spleen, muscle, ovary, and kidney, whereas along the digestive tract the relatively "higher" levels were found in rectum, followed by pyloric antrum, gastroesophageal junction and stomach fundus.

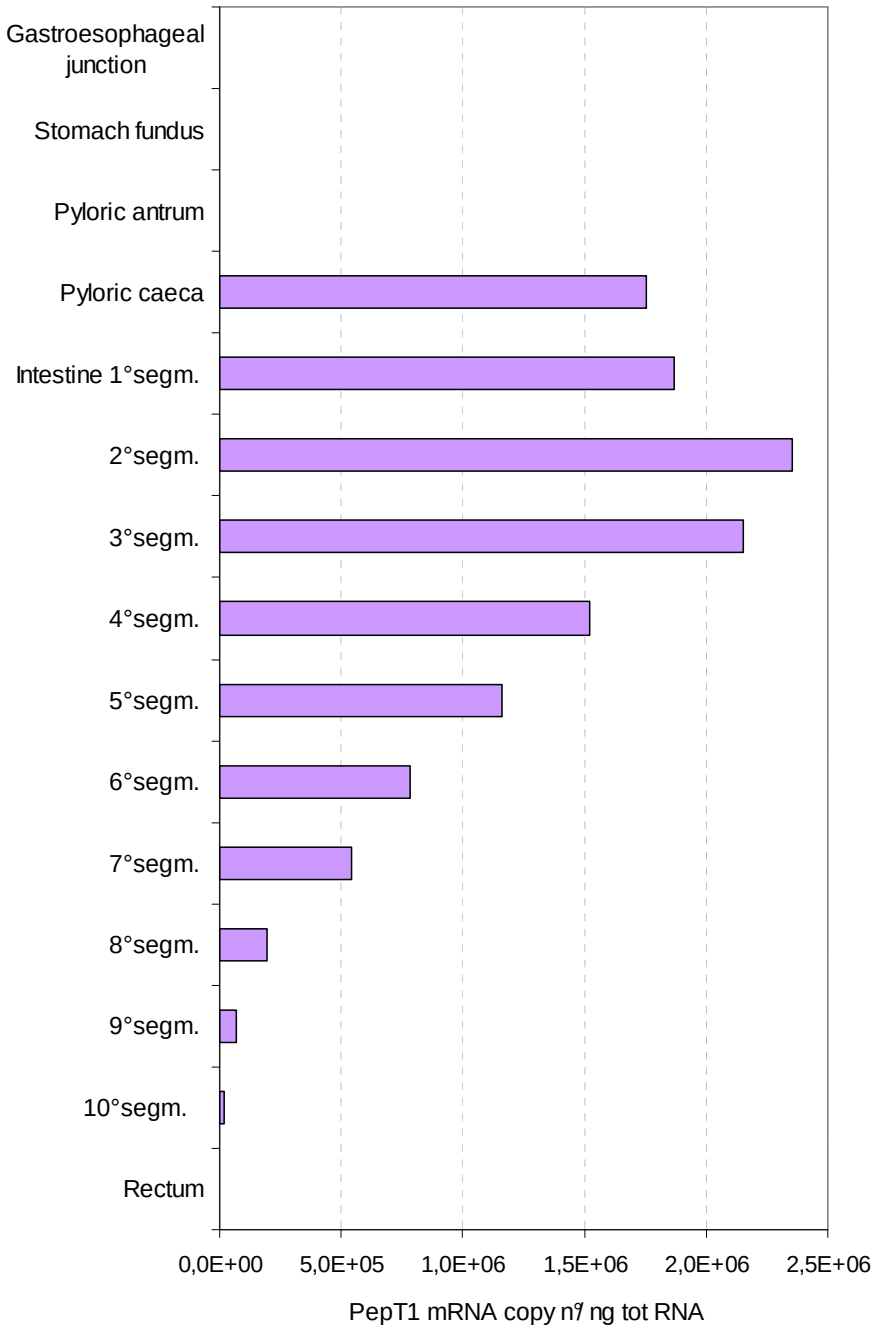

Figure 3.13 Spatial distribution of sea bass PepT1 mRNA in the digestive tract as determined by real-time quantitative PCR. Expression levels of PepT1 measured by real-time PCR in the different segments of sea bass digestive tract. PepT1 mRNA copy number was normalized as a ratio to 100 ng total RNA.

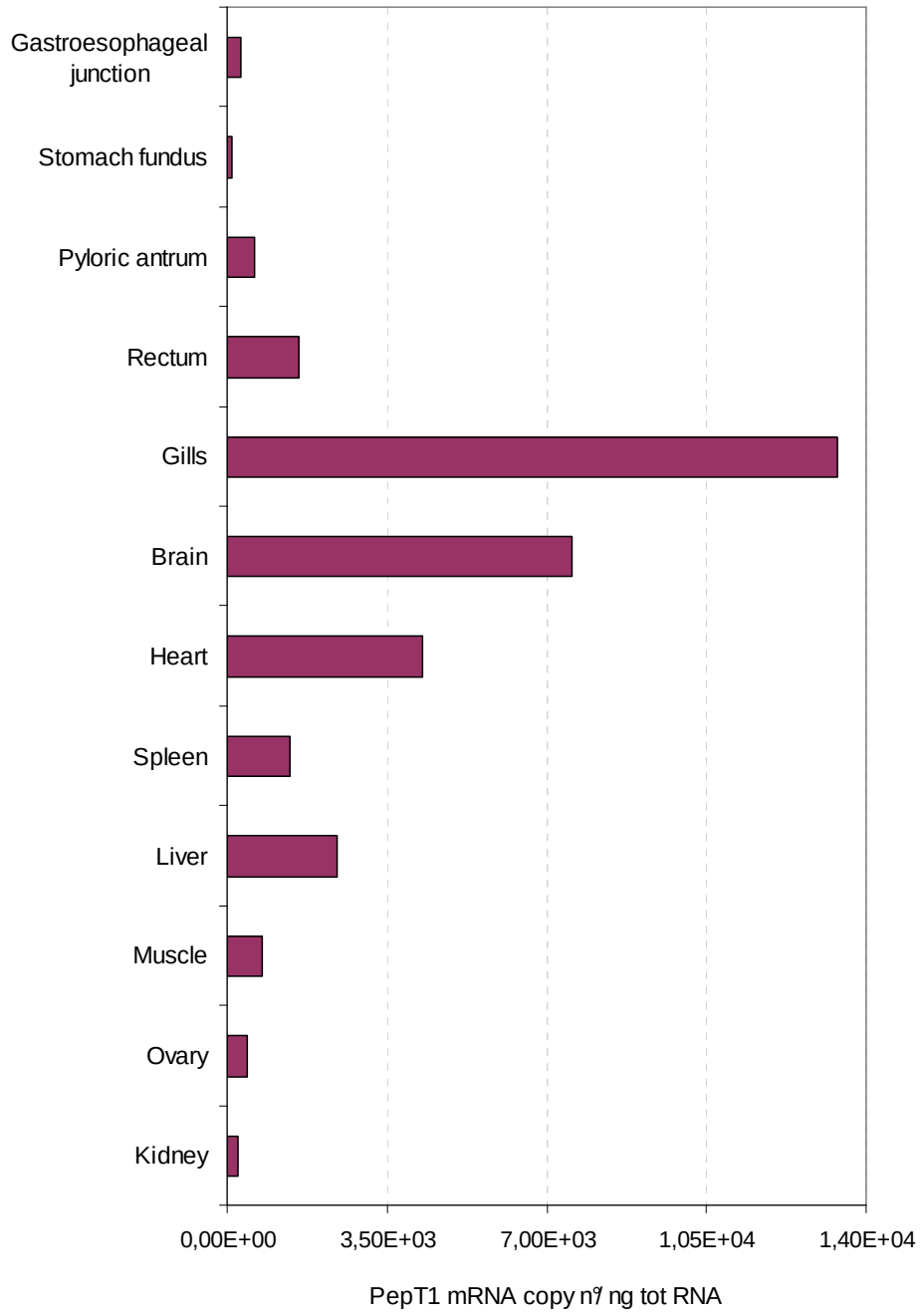

Figure 3.14 PepT1 gene expression in sea bass tissues as determined by real-time quantitative RT-PCR. PepT1 mRNA copy number was normalized as a ratio to 100 ng total RNA.

3.7 PepT1 mRNA copy number in sea bass proximal intestine during fasting and refeeding

The absolute mRNA levels of PepT1 in the proximal intestine in response to the feeding trial are presented in figure 3.15. Fasting for 35 days significantly decreased the amount of PepT1 transcripts ($P<0.05$) in the proximal intestine, whereas 4 days without feed did not contribute to a significant decrease in transcript levels as compared to controls. The recovery from fasting was associated with a significant increase in PepT1 mRNA transcript levels, compared to fasted animals, until the tenth day of refeeding. At the end of the fourth day of refeeding, the expression of PepT1 increased beyond control levels, in support of compensatory growth. Subsequently, 10 days after refeeding the PepT1 mRNA copy number levels decreased compared to the previous time point tested (4 day refeeding), remaining still significantly higher than the control values, and then returned to original levels after 21 days of refeeding (Fig. 3.15).

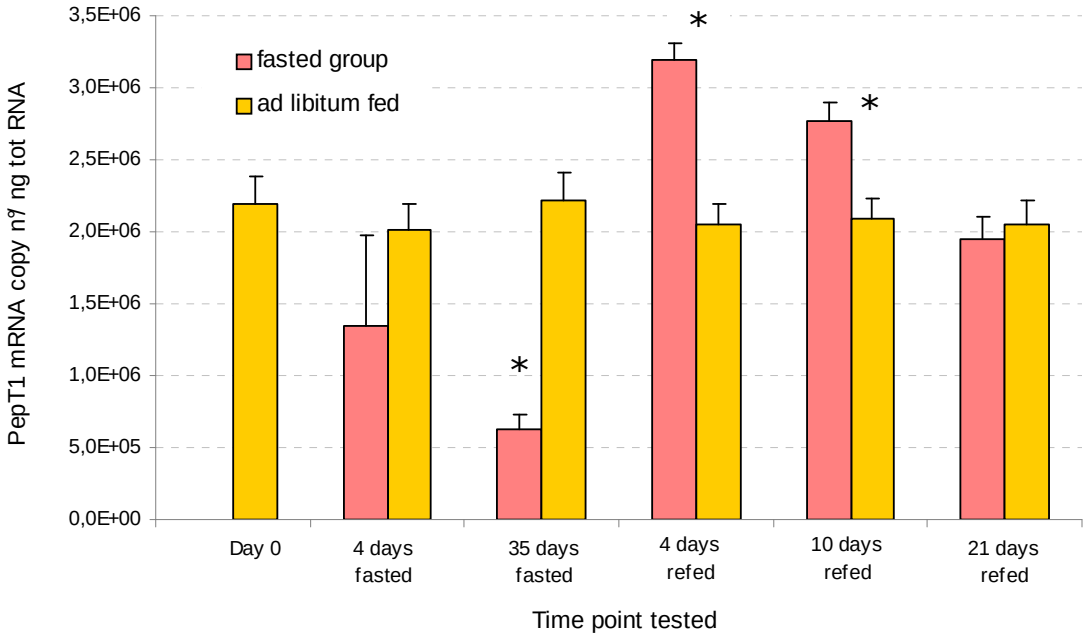

Figure 3.15 Expression levels of PepT1 measured by real-time PCR in sea bass proximal intestine in the course of experiment. PepT1 mRNA copy number was normalized as ratio to 100 ng total RNA. Fish were sampled before fasting (Day 0), 4 days after fasting (4 days fasted), at the end of fasting (35 days fasted), and then sequentially at 4, 14, 21 days following refeeding. The means of five animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). (*) indicate significantly different means from controls, for each time point tested ($P<0.05$).

4. Discussion

Fish tend to endure long-term starvation under certain physiological conditions and during particular stages of life. The physiological and biochemical mechanisms of starvation in fish have been studied intensively and have shown that fishes' ability to endure starvation and the mode by which they accommodate to starvation vary greatly (Valente et al., 1998; Jobling, 2006). The present study is part of our ongoing work on elucidating the genes involved in compensatory growth induced by fasting and refeeding in the European sea bass (*Dicentrarchus labrax*).

Accordingly, we have considered several genes involved in lipid metabolism, such as Δ6 desaturase, lipin, and PPAR. Little is known about the genomics of this species although it is a marine fish widely cultivated in the Mediterranean area, with desirable features for aquaculture.

Fatty acid delta-6 desaturase, the first gene considered, can introduce double bonds in selected positions of the acyl chains. In combination with elongation, desaturation results in the formation of a wide variety of fatty acids from a limited number of precursors that play a major role in lipid metabolism (Seiliez et al., 2001). We have isolated the complete cDNA sequence encoding for Δ6 desaturase in sea bass; this sequence shows a 80% homology with that of teleosts and 60% with that of amphibians, avians, and mammals, suggesting that its biological action may be well conserved through evolution.

This is the first study concerned with quantifying Δ6 desaturase mRNA in absolute terms by using real-time quantitative PCR after a long-term fasting and subsequent refeeding. Up to now, most studies have focused on desaturation activity protein in relation to nutritional modulation in teleosts. The major problem for fish nutrition is to fulfill the requirement of providing sources of 20-22-carbon polyunsaturated fatty acids (PUFAs) of the n-3 and n-6 series other than from fish oils originating in fisheries, which represent a limited resource (Sargent et al., 1999). The capacity to convert linoleic and linolenic fatty acids, abundant in vegetable oils, into C20 and C22 PUFAs is well established in some freshwater species, as seen for rainbow trout (Seiliez et a., 2001), but remains controversial in other cultured species, especially marine fish. In particular, Δ6 desaturase activity is rather limited in marine fish, and little is known about the possibility of enhancing Δ6 desaturase gene

expression in these species. Although long recognized, the reason for this deficiency in marine species is still the subject of controversy. Tocher et al. (2003a) showed that Δ6 desaturase is expressed at considerably lower levels in cod and other marine fish than in salmon, and this enzyme activity is much lower in tissues of cod compared to salmon (Tocher et al., 2006). In gilthead seabream, Δ6 desaturase was highly expressed in fish fed on a HUFA-free diet and only slightly expressed in fish fed on HUFA-rich diet, further demonstrating that this protein activity and HUFA synthesis are under strong control by dietary fats (Seiliez et al., 2003). Indeed, there is very strong evidence that the effects of dietary EPA and DHA are mediated through effects on Δ6 desaturase transcription. In addition, it has been well documented that lipid reserves are utilized preferentially over protein stores in the early stages of starvation to produce ATP (Navarro and Gutiérrez, 1995; Zhang et al., 2006). Although lipids cannot be used to synthesize carbohydrates, their oxidation can provide much of the ATP needed for carbohydrate synthesis and other functions. Therefore, research on lipid metabolism during starvation is particularly important. The strategy used for the present study was to assess fasting-induced changes in lipid metabolism, and to determine Δ6 desaturase mRNA expression in different tissues.

In sea bass liver, the mRNA copy number of this enzyme significantly decreased during the first 4 days of fasting as compared to controls, with a subsequent up-regulation after 35 days of fasting. These results confirmed that, except for the brief, initial period of transition (4 days), the organism adapts to optimal fasting in order to maintain physiological conditions for as long as possible, and that during prolonged starvation fatty deposits in the liver are broken down early, as has previously been shown in sea bass (Stirling, 1976), rainbow trout (Jezierska et al., 1982), and Arctic charr (Miglavs and Jobling, 1989a). In the absence of dietary lipids, liver synthesizes de novo the HUFAs required to cope with the fasting period. Accordingly, the significant increase in Δ6 desaturase transcript levels observed in liver after 35 days of fasting could be explained as being a consequence of intensive, highly unsaturated fatty acid biosynthesis. These results indicate that after starvation treatment, the hepatic fat of sea bass tends to be mobilized for energy, as it is the main energy source consumed by fish during prolonged fasting. Recently, Han et al. (2010) proved that tilapia responds to starvation by mobilizing

hepatic lipids, with a significant increase in the abundance of liver lipoprotein lipase mRNA, the enzyme that hydrolyzes stored triacylglycerols into free fatty acids, which are subsequently used for new synthesis of HUFAs.

On the other hand, Nakamura and Nara (2003) suggested that regulating phospholipid synthesis may represent the major physiological activity in liver, rather than triglyceride synthesis, because HUFA are mainly incorporated into phospholipids. They also proposed that the primary role of Δ6 desaturase in liver is to monitor and maintain cell membrane composition. Indeed, fish are poikilotherms, which are often subjected to temperature stress or seasonal temperature variation and which can exploit the diversity of lipid structure to fashion membranes to prevailing ambient temperatures in such a manner that they become more fluid in a cold-acclimatized state and less fluid in a warmth-acclimatized state (Dey et al., 1993). Consequently, the hepatic increase in Δ6 desaturase expression observed in sea bass may be also due to the raising need to synthesize phospholipids.

Liver Δ6 desaturase transcript content significantly declined during the first 4 days of refeeding and continued to decline until the tenth day, suggesting that sea bass could now synthesize HUFA from dietary lipid in reintroduced food rations via intestinal Δ6 desaturase activity. Gradually, desaturase expression levels return to values comparable with the control group at the end of the refeeding period.

In the proximal intestine, an opposite trend from that observed in liver has been shown, with down-regulation of Δ6 desaturase upon fasting and up-regulation during refeeding, results that are in line with the compensatory response. In particular, the significant decrease observed during fasting confirms the hypothesis that Δ6 desaturase is not transcribed in the absence of those dietary lipids necessary for HUFA biosynthesis in the gut. Subsequently, the fourth day of recovering from starvation was associated with a significant increase in Δ6 desaturase mRNA transcript levels, compared to control animals, suggesting that dietary lipids stimulate the expression of this enzyme. During refeeding, fish take advantage of the presence of food in order to optimize the intake of the nutrients required to compensate the reduced growth caused by fasting. Our results provide further evidence concerning the nutritional regulation of Δ6 desaturase expression and activity, as previously shown for gilthead seabream (Seiliez et al., 2003;

Izquierdo et al., 2008). According to all knowledge to date, HUFA are considered to have a regulatory function in the diet, and there is no evidence that starvation and subsequent refeeding affect the Δ6 desaturase mRNA levels in fish.

To summarize, our evidence supports the notion that, in sea bass liver, a dietary lipid deficiency increases the activity of long-chain fatty acyl desaturases for Δ6 desaturase, probably by forming adaptive enzymes. In contrast, in the proximal intestine dietary lipids may regulate desaturase enzyme expression, which appears to be depressed by fasting, but increases beyond normal levels in response to subsequent feeding. By utilizing fasting and refeeding as a method for producing a shortage of hepatic and intestinal HUFA, we were able to demonstrate directly that the Δ6 desaturase capability of long-chain fatty acid synthesis is suppressed or enhanced by dietary lipids.

In addition, since the lack of food is a basic prerogative for a compensatory process, an understanding of digestive machinery function during a fasting period could be a useful tool for investigating factors that limit the growth capacity of fish. In particular, investigating the expression and activity of metabolic enzymes of fish under these conditions can indicate to what point starvation can govern the utilization of nutrients after refeeding (Furné et al., 2008).

The next gene we have considered is lipin, a protein family member required for triacylglycerol (TAG) and phospholipid biosynthesis. Several studies have revealed a second function of lipin: it acts as a transcriptional coactivator that regulates expression of fatty acid utilization and lipid synthetic genes, in conjunction with peroxisome proliferator-activated receptor γ (PPARγ). PPAR are nuclear hormone receptors that control the expression of genes involved in mammalian lipid homeostasis, and previous studies on sea bass showed that the PPAR structure and expression profile in fish appears to be very similar to that of the mammalian PPAR homologs. Therefore, we isolated a partial amino acid sequence of sea bass lipin that is highly homologous with zebrafish lipin, which is also the only fish sequence present in the database. A complete coding sequence of sea bass PPARγ was already present in the database and it shows a high sequence similarity to that of other teleosts and vertebrate in general.

Then, we examined the mRNA expression levels of both of these genes in sea bass tissues. In the liver, the absolute mRNA levels of lipin and PPARγ followed the same

pattern, showing a significant increase after fasting for 35 days and a significant decrease at the end of 21 days of refeeding as compared to controls. Otherwise, the adopted food strategy did not affect lipin and PPARγ expression in the proximal intestine. A possible dietary influence on fish lipin expression has not been studied previously, and therefore our study can be considered the first to focus on the behavior of the transcripts of this gene during fasting and subsequent refeeding.

The increased lipin expression during starvation can be explained as a compensatory reaction to the increased demand for HUFA caused by the absence of dietary lipids, as already demonstrated for Δ6 desaturase liver expression. After reintroducing food and upon return to a normal nutritional status, lipin mRNA levels gradually decrease to the initial values, becoming even lower than those in controls.

As in mammals, lipin shows phosphatidate phosphatase activity with the appropriate properties and performs a regulatory function in glycerolipid synthesis, and starvation and stress factors increase this activity in the liver. Under these conditions, the liver receives an increased supply of stored fatty acids, which often exceeds its ability for β-oxidation (Brindley et al., 2009).

In sea bass, the liver responds to the fasting state by increasing its capacity for TAG and phospholipid synthesis by enhancing the lipin mRNA copy number. To confirm this, PPARγ transcript levels also increased, which supports the notion that these two genes belong to the same transcriptional regulation system. As described earlier, hepatic lipin mRNA expression is regulated by fasting and mediates PPARγ expression: increased lipin expression in hepatocytes amplified the expression of PPARγ target genes involved in fatty acid oxidation and suppressed expression of genes involved in de novo lipogenesis. In fact, starvation in fish promotes fatty acid oxidation and the reutilization of stored lipids to maintain the energy demand, as dietary lipids normally used for HUFAs biosynthesis are lacking.

Leaver et al. (2005) showed the same pattern in gilthead sea bream and plaice: here, hepatic PPARγ mRNA transcripts increased in the fasted state and decreased in the following refeeding period. In the intestine of these two species, no significant difference in PPAR isotype expression was observed, which is also in agreement with our results.

In mammals, PPARs activate transcription of target genes and play a critical role in the metabolic adaptation to starvation by inducing genes for fatty acid oxidation and increasing the degradation of HUFA in the liver (Nakamura and Nara, 2003). As a consequence, the requirement of HUFA for TAG and membrane phospholipid synthesis increased, also promoting Δ6 desaturase activity. Thus, the delayed induction of Δ6 desaturase by PPARs may represent a compensatory reaction to the increased demand of HUFA caused by the increase in oxidation and peroxisome proliferation, which is ultimately due to the fasting condition (Nakamura and Nara, 2003). Comparing these results with ours, we can confirm that the expression of all lipid metabolism genes considered is strongly influenced by fish nutritional status, suggesting that they are involved in the compensatory process.

In this study, we also isolated a full-length cDNA clone representing the oligopeptide transporter PepT1 in the European sea bass. All the following results are published in our article "Impact of feed availability on PepT1 mRNA expression levels in sea bass (*Dicentrarchus labrax*)" (Terova et al., 2009).

PepT1 is an integral plasma membrane protein responsible for the uptake of dietary di- and tripeptides in cells. The predicted PepT1 protein, like all members of the proton-coupled oligopeptide transporter (POT) superfamily, is predicted to traverse the membrane 12 times, with amino- and carboxyl- termini facing the cytosol, and with an elongated extracellular loop connecting the ninth and tenth transmembrane segments. Protein stretches with conserved residues are recognized as being structurally or functionally important within the mammalian PepT1 primary structure (Daniel and Kottra, 2004). One such protein stretch (PTR2 family proton/oligopeptide symporters signature 1' motif PROSITE pattern: PS 01022; amino acid residues 73-97 in sea bass PepT1) is located in the predicted end region of the MSH2 and the following intracellular loop, reaching into MSH3. The second, better conserved signature motif of the peptide transporter (PTR) family is found in the central part of MSH 5 and comprises a stretch of 13 amino acid residues (PTR2 family proton/oligopeptide symporters signature 2' motif PROSITE pattern: PS 01023; amino acid residues 166-178 in sea bass PepT1).

The predicted sea bass PepT1 amino acid sequence shows extensive sequence similarity to human and other vertebrate PepT1 in the first four amino-terminal transmembrane regions as well as in the regions from the seventh to ninth domain,

whereas there are variations in the large extracellular loop between transmembrane domains 9 and 10. In fact, the length of this loop is 200 amino acid residues in sea bass, 187 aa in cod, 240 aa in China rockfish, 148 aa in zebrafish, 242 aa in frog, 205 aa in dog, 204 aa in chicken, 203 aa in sheep and cattle, 236 in turkey, 239 in rabbit, 204 in monkey, human and pig, and 240 aa in rat and mouse.

To address the structure-function properties of mammalian peptide transporters, Doring et al. (1996), Fei et al. (1997), and Terada et al. (2000) generated a range of chimeric transporters in which regions of mammalian PepT1 were replaced with the corresponding regions from PepT2 and *vice versa*. The distinctly different affinities for the same substrates of PepT1 and PepT2 were used to define the obtained phenotype and to draw conclusions about important protein regions. The functional characteristics of these chimeras were studied after they were expressed in *Xenopus* oocytes. These experiments suggested that the first four amino-terminal transmembrane regions and also MSH 7 to 9 are regions important for substrate binding and in which most PepT1 functions are encoded. The role of the large extracellular loop between transmembrane domains 9 and 10 is unknown, and only one highly conserved residue (E595, numbering according to human PepT1) in this C-terminal half of PepT1 has proven to be of functional relevance (Bolger et al., 1998).

Several functionally important amino acid residues and sequence motifs, described in all known vertebrate PepT1 proteins, are also found in the sea bass (see Fig. 3.4 and 3.5 in the section Results). They include: the highly conserved tyrosine residue (Y12) on the first membrane-spanning helix; the highly conserved histidine residue (H57) on the interface of the second transmembrane domain and the extracellular side; the H57 adjacent tyrosine residue (Y56); the tyrosine residue predicted to lie in the second transmembrane domain (Y64); Tyr-167 in the fifth transmembrane domain; His-121 and His-260; the highly conserved Ser-164, Leu-168, Gly-173, Ile-179, Asn-171, Ser-174, and Pro-182; Arg-282, which in sea bass and other teleosts is replaced with another positively charged amino acid (Lys); Tyr-287 and Met-292, Trp-294, Lys-296, and Phe-297; and the highly conserved Asp-341, Pro-586, and Glu-595. The overall high degree of PepT1 sequence conservation through evolution is not only consistent with its essential role for growth and metabolism,

but also suggests that its biological action may be equally well conserved. In fact, numerous aspects of digestion and absorption in fish and mammals are similar, demonstrating high conservation of these mechanisms during evolution.

Digested dietary proteins in both teleosts and mammals are subject to hydrolysis by a range of proteases and peptidases that generate a mixture of free amino acids and small peptides which are efficiently absorbed across the apical membranes of enterocytes (Clements and Raubenheimer, 2006). Cellular transport of amino acids and small peptides is thus a key, final step in their assimilation by the intestine. Two distinct types of brush border membrane-associated transport systems are involved in this process: (1) amino acid transport, which may or may not be sodium dependent; and (2) transport of small peptides (2-3 amino acids) coupled to a H^+ gradient. Intestinal peptide transport is of major nutritional significance in that the intraluminal products of protein digestion are predominantly di- and tripeptides, not amino acids, as was widely believed over 30 years ago (Adibi, 2003). In addition, many amino acids are more rapidly and efficiently absorbed in peptide form (Gilbert et al., 2008a). Furthermore, the high activity of intracellular peptidases showing strict specificity for the hydrolysis of di- and tripeptides suggests that di- and tripeptides peptides, but not larger ones, may be absorbed in intact form and that free amino acids are then released intracellularly (Gonçalves et al., 2007; Kottra et al., in press) However, the extent to which a di- or tripeptide that is released during digestion is finally broken down at the brush border membrane or is taken up into the cell and hydrolyzed there is not known.

Although results from many studies demonstrated that intestinal cells take up peptides, the transporter responsible for this, designated PepT1, was not identified until 1994: Fei et al. cloned PepT1 by microinjecting mRNA isolated from rabbit intestine into *Xenopus* oocytes, which resulted in functional expression of the protein. In subsequent years PepT1 was characterized in great detail in higher vertebrates, mostly in mammals, but also in birds (Daniel et al., 2006).

At the beginning of this study, information about this gene in lower vertebrates such as teleosts was limited, with the exception of PepT1 of the zebrafish, Atlantic cod, Asian weatherloach, and China rockfish. In these species the functional activity and tissue expression pattern of PepT1 have been assessed (Verri et al., 2003; Rønnestad et al., 2007b; Gonçalves et al., 2007). In recent years, PepT1 has been

studied in an increasing number of fish species, such as icefish, *Chionodraco hamatus* (Maffia et al., 2009); crucian carp, *Carassius carassius* (accession no. HM453869); rainbow trout (Ostaszewska et al., 2010); and Atlantic salmon, *Salmo salar* (Rønnestad et al., 2010). Finally, we isolated PepT1 sequence in yellow perch, *Perca flavescens* (accession no. GQ906471), and gilthead seabream, S*parus aurata* (accession no. GU733710).

Peptide transport has also been well demonstrated in teleosts, where it occurs via carrier-mediated mechanisms, and is highly stimulated by an inside-negative transmembrane electric potential (Thamotharan et al., 1996; Maffia et al., 1997). Proton/glycyl-sarcosine (Gly-Sar) cotransport has been described in the brush border membrane of the absorbing cells in tilapia intestine and rockfish (*Sebastes caurinus)* intestine and pyloric ceca (Thamotharan et al., 1996). Moreover, H^+/D-phenyl-L-alanine cotransport has been described in eel intestinal brush border membrane (Verri et al., 1992, 2000; Maffia et al., 1997). In a complementary study (Sangaletti et al., 2009), we recently examined the electrophysiological behavior of *Xenopus* oocytes injected with cRNA transcripts obtained from our cloned sea bass PepT1 (Terova et al., 2009), with the two-electrode voltage-clamp technique: its properties conform in most aspects to the observations reported for the zebrafish PepT1, the only other teleost PepT1 that has been functionally characterized (Verri et al., 2003), and more generally to those of mammalian transporters (Daniel et al., 2006). The main characteristic fully conforms to the findings in the other PepT1s is that transport-associated currents were observed when various di- or tripeptides were applied to oocytes, while no currents were generated by histidine nor by other single amino acids. Sea bass PepT1 also exhibited presteady-state currents in the absence of substrates. Moreover, Michaelis–Menten analysis of the transport currents showed an increase in apparent substrate affinity at acidic pH, which was very similar to that exhibited by the related transporter from zebrafish (Verri et al., 2003) but in contrast from that described in other species.

In the course of the past few years, the study of intestinal peptide transport has rapidly evolved into a field of exciting nutritional and biomedical applications. In particular, the effect of dietary protein on PepT1 expression and activity has been an area of active research in recent years. Results from these studies (Gilbert et al.,

2008a; Daniel, 2004) demonstrated that PepT1 expression levels and function are very responsive to dietary treatments. The corresponding information for fish is completely unknown; however, this would be of great importance in particular for the farmed species raised in feed-based aquaculture systems. From the commercial perspective, it is very costly to use fish dietary protein as a nutrient, and even fractional improvements in this area have the potential to save the aqua-feed industry millions of euros and also to reduce the amount of nitrogen excreted into the environment. Accordingly, we utilized real-time PCR technology to carefully assess the impact of dietary manipulation on PepT1 mRNA expression levels in several sea bass tissues. Our results demonstrate that PepT1 is abundantly expressed in the proximal intestine and pyloric ceca of this species, but it is virtually absent or expressed at very low levels in the stomach and in the distal part of intestine. Thus, it is also likely that, in sea bass, the proximal intestine is the main expression and production site of PepT1, as also demonstrated in humans (Ford et al*.,* 2003) and other fish species, such as cod (Rønnestad et al., 2007), weatherloach (Gonçalves et al., 2007), and zebrafish (Verri et al., 2003). The presence of PepT1 mRNA in pyloric ceca and in the subsequent 10-cm region of the intestinal tissue, at albeit differing levels, is similar to the spatial distribution of PepT1 mRNAs found in Atlantic cod intestine (Rønnestad et al., 2007). In addition, the relatively very low level of expression in sea bass gills, heart, liver, and stomach matches the pattern reported for Atlantic cod (Rønnestad et al., 2007). Sites with lower levels of PepT1 expression or activity, or both, are also found in human colon and bile duct epithelium and in rat brain and liver (Miyamoto et al., 1996; Knutter et al., 2002; Ford et al., 2003)

The study of the dietary regulation of this transporter in the proximal portion of the intestine revealed that PepT1 mRNA levels had not changed after 4 days of fasting, but were down-regulated after 35 days of fasting. These results do not match those of previous studies that investigated the behavior of the transcripts of this gene over brief fasting periods in mammals or in birds. Short-term starvation in rats has been shown to increase mRNA and protein expression of PepT1: in the study of Ihara et al. (2000), mRNA and protein expression were increased by 179% in rats starved for 4 days, whereas in the study of Thamotharan et al. (1999), PepT1 mRNA and protein increased 3-fold, and the rate of peptide transport increased

dramatically after only 1 day of fasting. Similarly, Gilbert et al. (2008b) found that feed restriction increased expression of PepT1 mRNA in broilers aged 3-14 days after hatching.

The different fasting-induced changes in PepT1 mRNA expression in sea bass may reflect the impact of a more substantial fasting period in fish as compared to mammals and birds. Maybe the duration of fasting in the studies conducted in rats and birds was not enough long to generate the magnitude of nutritional insult necessary to down-regulate PepT1 mRNA.

Short-term refeeding (4 days) promoted a remarkable increase in the transcription levels of sea bass PepT1 mRNA, which significantly exceeded the levels of the controls. This result might be explained by the great quantity of protein taken in with the food in the initial days of refeeding. In fact, refeeding of fish after a long starvation period was marked by hyperphagia as early as the first day. In our experiment, fish were fed more avidly and grew faster than the controls after the period of fasting, compensating for lost body weight in an apparent attempt to catch up with conspecifics that had continuous access to food. Generally, we can observe that fish recovered the depressed growth phase induced by fasting, increasing the intake of food during the refeeding period, and optimized protein intake, enhancing PepT1 expression levels in the intestine.

Hyperphagia was the main mechanism by which sea bass compensated for their growth loss during fasting. The significant increase in food intake promoted rapid growth, but no improvement in feed efficiency was observed.

Experiments in which juvenile three-spined sticklebacks were exposed to 1 or 2 weeks of starvation have also demonstrated full growth compensation (Zhu et al., 2001). The compensatory growth response reflected the hyperphagia in the deprived groups, with a higher consumption on the first day of refeeding, as we showed for sea bass. In several studies, food consumption of individual fish was monitored during a phase of compensatory growth, and hyperphagia was always observed (Miglavs and Jobling, 1989; Russell & Wootton, 1992; Hayward et al., 1997; Xie et al., 2001).

Different models (Hubbell, 1971; Broekhuizen et al., 1994) have assumed that there is an optimum trajectory for growth in mass, and any deviations from that trajectory evoke a compensatory change in food consumption to reduce the

magnitude of the deviation. Moreover, the duration of compensatory responses, such as hyperphagia, are correlated to the intensity of the growth depression.

After a long fasting period, like that in our study, quite long times appear to be required for the target genes transcripts to return to "normal". In fact, the levels of mRNA only reached the control mRNA levels at the end of the third week of refeeding. Perhaps the extent to which fasted fish display high levels of gene expression upon return to adequate feeding conditions is related to the severity of the nutritional insult sustained by the animals. To confirm this hypothesis in fish additional studies are needed as, to our knowledge, this is actually the first one to have investigated Δ6 desaturase, lipin, and PepT1 expression patterns for such a long fasting period. However, we cannot extensively discuss the potential role of the fasting-induced changes in Δ6 desaturase, lipin, PPARγ and PepT1 mRNA levels based on the results of the present study as we do not know whether the mRNA profile is consistent with the functional protein levels. Therefore, without protein data we cannot draw any conclusions about the relationship between the mRNA levels and the protein activity in the intestine and in the liver of fasted sea bass. Thus, our aforementioned hypothesis, together with the notion that the different fasting-induced changes in all target gene mRNA expression may also reflect differences in energy metabolism between homeoterms (mammals and birds) and poikilotherms (fish), must be confirmed by further investigations.

Lastly, fasting in our study was associated with significant changes in two of the growth indexes examined: body weight and condition factor (an indicator of body shape), which suggests that animals were duly stressed (Terova et al., 2008). We observed that sea bass growth follows the idealized pattern of growth compensation based on Jobling (1994). The strain of long starvation in sea bass also modifies the growth response after the food supply has been resumed. Fasted fish showed a significant increase in body weight at the end of 35 days of fasting compared to controls, and the entire refeeding period was needed to regain their normal body weight. This result further confirms that fasting and subsequent refeeding elicited a compensatory response in sea bass.

4.1 Conclusion

We have isolated the cDNA encoding sequences of all selected genes in sea bass, and we also demonstrated that the nutritional state of the animal influences gene expression levels, inducing down-regulation or up- regulation during fasting and refeeding depending on the specific gene considered. Future studies are necessary, however, to completely clarify the underlying mechanism of these target genes action in sea bass, also measuring physiological effects produced by the proteins. Indeed, the present study is the first one to investigate the behavior of the transcripts of these genes over such a long period of fasting and subsequent refeeding.

The findings of the present work could have important implications for aquaculture. In fact, under farming conditions routine manipulation and excessive crowding of animals are key factors in disturbing the feeding behavior of fish (Ashley, 2007). Physical stress induces a reduction in food intake which may last several hours or even days (Bernier, 2006; Kestemont and Baras, 2001). Recently, Rubio et al. (2010) demonstrated that sea bass feeding activity is extremely sensitive to stress and it is reduced by the influence of human interference; when the disturbance stopped, fish show an increased food intake. This feeding behavior resembles the same pattern observed in a compensatory growth phenomenon, such as during our experimental study. More attention should be directed to sea bass nutrition and to investigating the mechanisms that regulate compensatory growth, which could provide more new information applicable to aquaculture farms. In particular, it may be useful to assess the feeding response to acute stressors to ensure fish welfare. Moreover, subjecting fish to a fasting period might be more efficient and economical so as to avoid giving an unnecessary supply of feed, as partial or total deprivation of food and subsequent refeeding seem to be favorable for recovering appetite and growth.

Finally, we have also identified molecular markers whose expression is affected by a stress factor that can be found under natural conditions. This evidence underscores the need to direct even more the attention to learning how aquaculture economic imperatives can be combined with the need to protect fisheries and safeguard biodiversity.

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